PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Curtis C. Harris *et al.*

Application No.: 10/633,789

Filed: August 4, 2003

For: SCREENING ASSAYS FOR COMPOUNDS THAT CAUSE APOPTOSIS

Confirmation No.: 9707

Examiner: Anish Gupta

Art Unit: 1654

Declaration of Xin Wei Wang Under 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Xin Wei Wang, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my knowledge are true and statements made on information or belief are believed to be true.

2. I hold B.S., M.S., and Ph.D. degrees from Shanghai Medical University, Chinese Academy of Sciences, and New York, earned in 1982, 1984, and 1991, respectively. Currently, I am a Senior Investigator and Head of the Liver Carcinogenesis Section, Laboratory of Human Carcinogenesis, NCI, NIH, Bethesda, MD, and has been in this and related positions since 1995. A true copy of my Curriculum vitae is attached as Exhibit A.

3. The present invention is directed to methods for identifying compounds that modulate XPB or XPD helicase-dependent p53-mediated apoptosis by providing a biologically active p53 polypeptide and a XPB or XPD helicase polypeptide, contacting a compound suspected of inducing XPB or XPD helicase-dependent p53-mediated apoptosis with the XPB or XPD and p53 polypeptides, and detecting whether or not the compound is capable of specifically inhibiting binding of the p53 polypeptide to the XPB or XPD helicase, wherein a compound that specifically inhibits the binding of the p53 polypeptide to the XPB or XPD helicase is a compound that modulates helicase-dependent p53-mediated apoptosis.

4. I am a named co-inventor on the above-referenced application. I have read and are familiar with the contents of the subject patent application. I have also read the Office Action received from the United States Patent and Trademark Office dated June 7, 2007. It is my understanding that the Examiner is alleging that the claimed invention is anticipated by the White and Reed patents (U.S. Patent Nos. 5,604,113 and 5,484,710; "White" and "Reed", respectively). More particularly, the Examiner alleges that XPB or XPD helicase binding to p53 is an inherent feature of p53-mediated apoptosis. I further understand that the Examiner has indicated that the anticipation rejection can be overcome by a showing that the art recognized other mechanisms by which p53 mediates apoptosis.

5. This declaration is provided to demonstrate that it was known in the art at the time the application was filed that the p53 protein can mediate apoptosis through multiple mechanisms. In particular, this declaration presents evidence demonstrating that p53-mediated apoptotic events can proceed through helicase-independent mechanisms.

There are multiple mechanisms of p53-mediated apoptosis

6. As indicated by the review article "Control of apoptosis by p53" (Fridman, J.S. and Lowe, S.W., *Oncogene*, 22: 9030-9040 (2003); "Fridman", provided as Appendix B), it is known in the art that p53-mediated apoptosis proceeds through both transcription-dependent and transcription-independent mechanisms (see Abstract).

7. The understanding that p53-mediated apoptosis proceeds through multiple mechanisms, involving transcriptional and non-transcriptional mechanisms, has been further strengthened by studies conducted since Fridman's 2003 review article. For example, the more recent review article "Dissecting p53-dependent apoptosis" (Chipuk, J.E. and Green, D.R., *Cell Death and Differentiation*, 13: 994-1002 (2006); "Chipuk", provided as Appendix C) provides a clear delineation of transcription-dependent and transcription-independent mechanisms of p53-mediated apoptosis in Figure 2. As shown in Figure 2 of Chipuk, the transcription-dependent mechanism of p53-mediated apoptosis entails the transcriptional activation of pro-apoptotic genes such as BAX, NOXA, PUMA, BID, CD95, APAF-1, DR5, p53AIP1, among others. Figure 2 also indicates that a transcription-independent mechanism of p53-mediated apoptosis entails the extranuclear function of p53 on mitochondrial outer-membrane permeabilization (MOMP) through the direct binding of p53 to anti-apoptotic proteins such as members of the Bcl family. Thus, it is known in the art that p53-mediated apoptosis proceeds through transcription-dependent and transcription-independent mechanisms.

Helicase binding to p53 is only one mechanism for p53-mediated apoptosis

8. The invention is based on the identification of a previously unknown mechanism by which p53-mediated apoptosis occurs. Specifically, we found that the XPD or XPB helicases bind to the C-terminal domain of wild type p53 (see, e.g., Figure 2B of the subject application). Upon binding of the XPB or XPD helicases to p53,

helicase activity is inhibited (see, e.g., Figure 5C of the subject application). The cellular consequence of helicase binding to p53 is the induction of apoptosis (see, e.g., Example 8 and Table 2 of the subject application). The data in Table 2 indicates that cells with defective XPB or XPD helicases are resistant to p53-induced apoptosis, demonstrating that one mechanism for apoptosis involves helicase-binding to p53 in apoptosis.

9. Further studies conducted under my supervision confirmed a role for XPB or XPD helicase binding to the C-terminal domain of p53 in the induction of apoptosis. This work is described in Zhou *et al.*, *Cancer Research*, 59: 843-848 (1999) ("Zhou", provided as Appendix D). Three C-terminal p53 mutants ("342-stop", "360-del", and "387-del") were expressed in cells and assayed for apoptosis. Wild type p53 was used as a control. The "342-stop" mutant has a deletion of both the C-terminal domain XPD/XPB binding site and the p53 transcriptional transactivation domain. The "360-del" mutant has a deletion of the C-terminal domain XPD/XPB binding site, while retaining the p53 transcriptional transactivation domain. The "387-del" mutation has a small C-terminal deletion that retains the XPD/XPB binding site, while also retaining the p53 transcriptional transactivation domain.

10. The results of this experiment demonstrate that C-terminal p53 mutants with deletions of the XPD/XPB binding site (i.e., "342-stop" and "360-del") displayed a significantly reduced ability to induce apoptosis (see Figure 3 and Table 1 of Zhou). In contrast, the "387-del" mutant, which retains the XPD/XPB binding site displayed a more modest reduction in apoptosis. These results demonstrate that XPD or XPB helicase binding to the C-terminal of p53 is one mechanism for p53-mediated apoptosis.

Loss of helicase binding to p53 does not abolish p53 induction of pro-apoptotic genes

11. As explained in detail in paragraph 6 above, one well known mechanism for p53-mediated apoptosis is through the induction of pro-apoptotic gene expression. Thus, as described in Zhou, we also tested the series of C-terminal mutants of p53 for their ability to induce expression of a number of known p53 target genes, including the well characterized pro-apoptotic gene, *Bax*. Our results, as shown in Figure 5 and Table 1 of Zhou, indicate that p53 mutants which have deletions of the XPD/XPB binding site ("360-del"), but which retain their transcriptional transactivation domains, maintain their ability to induce the expression of the pro-apoptotic gene, *Bax*. The "342-stop" mutant lacks both the transcriptional transactivation domain and the XPD/XPB binding site, and thus, fails to induce the expression of *Bax*.

12. The experiments in Zhou unequivocally demonstrate that p53 binding to XPD or XPB helicase and p53 induction of pro-apoptotic genes, such as *Bax*, are distinct mechanisms by which p53 effects apoptosis in cells. In particular, I conclude that transcription-dependent p53 induction of pro-apoptotic genes is at least one mechanism of p53-mediated apoptosis that is independent of p53 binding to XPD or XPB helicase.

13. In view of the foregoing, it is my scientific opinion that multiple mechanisms for p53-mediated apoptosis, including mechanisms that do not involve XPB or XPD helicase binding to p53, are known in the art.

Dated: 8/30/07 By: _____
Xin Wei Wang, Ph.D.

CURRICULUM VITAE

Name: Xin Wei Wang, Ph.D.

Date and Place of Birth: April 21, 1957; Shanghai, China

Citizenship: United States of America

Marital Status: Married to Lisha Xu with two children (Anran and Jennifer)

Education:

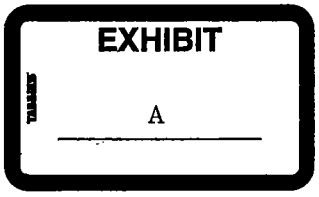
1982	B.S., Shanghai Medical University, Shanghai, China
1984	M.S., Chinese Academy of Sciences, Shanghai, China
1991	Ph.D., New York University, New York, NY

Brief Chronology of Employment and Training:

1982-1985	Graduate Research Assistant in Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China
1985-1986	Research Fellow in Department of Pharmacology, The University of Texas, Health Science Center at Houston, Houston, TX
1986-1987	Research Scientist in Institute of Environmental Medicine, New York University Medical Center, New York, NY
1987-1991	Graduate Research Assistant in the Institute of Environmental Medicine, NYU Medical Center, New York, NY
1991-1992	Postdoctoral Fellowship in Roche Institute of Molecular Biology, Nutley, NJ
1992-1995	IRTA, Laboratory of Human Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, MD
1995-1998	Senior Staff Fellow, Laboratory of Human Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, MD
1998-2005	Investigator and Head, Liver Carcinogenesis Section, Laboratory of Human Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, MD
2002-Pres.	Adjunct Associate Professor, Department of Pathology, University of Maryland Cancer Center, Baltimore, MD
2005-Pres.	Senior Investigator and Head, Liver Carcinogenesis Section, Laboratory of Human Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, MD

Societies:

American Association for Cancer Research (member)



American Association for the Advancement of Science (member)
CAST-Biomedical/Pharmaceutical Society (board of director)
CCR-NCI HIV and Cancer Virology Faculty (members)
The International Society of Gastroenterological Carcinogenesis (Board of Directors)

Faculty Membership:

CCR-NCI Cellular, Molecular and Developmental Biology Faculty
CCR-NCI Genetics, Genomics, and Proteomics Faculty
CCR-NCI Gene Expression Faculty
CCR-NCI Bioinformatics, Biostatistics and Computational Biology Faculty
CCR-NCI Molecular Targets Faculty
Gastrointestinal Malignancies Working Group
Metastasis Working Group

Honors, Distinguished Lectures and Other Special Scientific Recognition:

Recipient of Best student awards from Shanghai First Medical College, 1980-1981
Graduate fellowship award from Chinese Academy of Sciences, 1982-1985
Recipient of Best thesis (MS) award from Committee of Pharmaceutics Sinica, Shanghai Branch, 1984
Member of Graduate Student Council in Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 1983-1984
Graduate research assistantship from New York University Medical Center, 1987-1991
Recipient of a student travel award from American Society of Toxicology for attending annual meeting in 1988
Recipient of Graduate student award for meritorious research presented by the Carcinogenicity Specialty Section, Society of Toxicology, 1991
Invited lecture, NCI, NIH, Bethesda, MD, 1992
Invited speaker to the Life Sciences Symposium on Human Genetics, Association for Chinese Scientists in America, the Great Washington Chapter, 1993
Invited lecture, INSERM Unit, University of Strasbourg, Strasbourg, France, 1994
Invited lecture, Department of Cell Biology and Genetics, Medical Genetics Center, Erasmus University, Rotterdam, Netherlands, 1994
Invited speaker, Symposium on DNA Repair and Human Syndrome, NIH Research Festivals, 1994
Invited speaker, DNA repair interest group, NIH, Bethesda, MD, 1995
Invited speaker, Genetic Susceptibility and Molecular Carcinogenesis, AACR, Keystone, 1996
Invited speaker, 1996 Shanghai International Symposium on Liver Cancer & Hepatitis, Shanghai, China, 1996
Invited lecture, Department of Pathology & Oncology, University of Maryland Cancer Center, Baltimore, MD, 1996
Invited speaker, IASLC Workshop, Nancy, France, 1996

Invited speaker, symposium on apoptosis, Scanning Microscopy International, Chicago, IL, 1997
Invited speaker, Cancer genetics and tumor suppressor genes conference, Frederick, MD, 1997
Invited lecture, National Cancer Institute, Bethesda, MD, 1997
Invited speaker, XVIII International Congress of Genetics, Beijing, China, 1998
Invited speaker, the 5th International Symposium on Dendritic Cells in Fundamental Clinical Immunology, Pittsburgh, PA, 1998
Invited lecture, The National Capital Area Branch of the Society for In Vitro Biology, Beltsville, MD, 1998
Recipient of NIH Technology Transfer Awards, 1998, 2000, 2002-2005
Invited speaker, the 14th Aspen Cancer Conference, Aspen, CO, 1999
Invited speaker, Symposium on DNA Repair and Apoptosis, NIH Research Festivals, 1999
Invited lecture, Department of Biochemistry and Molecular Biology, University of Maryland, Baltimore, MD, 2000
Invited lecture, Department of Pathology, Virginia Commonwealth University, Richmond, VA, 2000
Invited speaker, the 9th International Congress of Toxicology, Brisbane, Australia, 2001
Invited speaker, the 16th Aspen Cancer Conference, Aspen, CO, 2001
Invited lecture, Multicenter Hemophilia Cohort Study-II, Washington, DC, 2002
Invited speaker, Molecular Genomics 2002: profiling of gene expression, Galveston, TX, 2002
Lecture, Graduate Class for Topics in Molecular Epidemiology, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC, 2002
Lecture, Clinical Center, NIH, Bethesda, MD, 2002
Invited speaker, FASEB Summer Research Conference on "Mechanisms of Liver Growth, Differentiation and Molecular Pathogenesis of Hepatic Diseases, Snow Mass, CO, 2002
Organizer, International Workshop on Human Hepatocellular Carcinoma, Bethesda, MD, 2002
Invited lecture, Liver Cancer Institute and Zhongshan Hospital, Fudan University, Shanghai, China, 2002
Invited lecture, Cancer Institute/Hospital, Chinese Academy of Medical Sciences, Beijing, China 2002
Invited lecture, Thomas Jefferson University, Jefferson Center for Biomedical Research, Doylestown, PA, 2002
Invited lecture, Bernie Carter Center for Immunology Research, University of Virginia Health Sciences Center, Charlottesville, VA, March 2003
Invited lecture, Graduate Class for Topics in Molecular Epidemiology, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC, May 2003
Invited lecture, The Thirteenth International Symposium of Hiroshima Cancer Seminar, Hiroshima, Japan, October 2003
Invited lecture, Symposium on Molecular Diagnosis of Human Cancer, Sponsored by Shanghai Medical Association, Shanghai, China, November 2003
Invited lecture, The GW Cancer Institute, The George Washington University Medical Center, Washington, DC, January 2004

Invited lecture, Hong Kong Shanghai International Liver Congress 2004, Hong Kong, China, February 2004
Invited lecture, The 19th Aspen Cancer Conference, Aspen, Colorado, August 2004
Invited lecture, The 3rd International Conference on Gastroenterological Carcinogenesis, Sapporo, Japan, August 2004
Invited lecture, The 35th Environmental Mutagen Society Annual Meeting, Pittsburgh, Pennsylvania, October 2004
Guest speaker, Division of Gastroenterology and Liver Diseases, Mount Sinai School of Medicine; New York, New York, October 2004
Keystone Symposia Program Committee Meeting (as an ad hoc member), Keystone, Colorado, January 2005
Distinguished lecturer, Frontiers in Oncology Seminar Series: "Progress on molecular diagnosis and molecular targets for human hepatocellular carcinoma". University of Maryland Greenebaum Cancer Center, Baltimore, MD, March 2005
Invited speaker, The National Cancer Institute Liver Cancer Symposium, Bethesda, MD, April 2005
Invited speaker, The 96th Annual Meeting of the American Association for Cancer Research, Anaheim, CA, April 2005
Invited speaker and co-organizer, NCI Symposium, Shanghai-Hong Kong International Liver Congress 2006, Shanghai, China, March 2006
Invited speaker, CNIO Cancer Conference, Madrid, Spain, May 2006
Invited speaker, 4th International Society of Gastroenterological Carcinogenesis Conference, Hawaii, August 2006

Administrative Service:

Institutional Service

NCI-UMD Tissue Resource Technical Review Panel, 2000, 2005
NIH FARE Committee, 2001-2002, 2004-2005
LHC Microarray Interest Group, 2002-2004
NIH-NCI HCC Clinical Steering Committee, 2005-Pres.
CCR-NCI Inflammation and Cancer Initiative Discussion, 2005

National Service

Organizer, International Workshop on Human Hepatocellular Carcinoma, Bethesda, Maryland, 2002
The International Society of Gastroenterological Carcinogenesis (Board of Directors), 2003-2005
NRC Research Advisor, 2005-Pres.
Co-organizer, NCI- cosponsored Symposium on liver cancer at the Shanghai-Hong Kong Liver Congress, March 2006

Reviewer for the following Scientific Journals: (1995-Present)

American Journal of Pathology
Cancer
Cancer Detection and Prevention
Cancer Epidemiology, Biomarkers & Prevention
Cancer Genetics and Cytogenetics
Cancer Letters
Cancer Research
Carcinogenesis (**Editorial Board**), 2002-2005
Clinical Cancer Research
Gastroenterology
Genes and Development
Experimental Cell Research
FEBS Letters
Hepatology
Immunity
Journal of Biological Chemistry
Journal of Clinical Investigation
Journal of Hepatology
Journal of National Cancer Institute
Molecular and Cellular Biology
Molecular Cancer Research
Molecular Carcinogenesis
Neoplasia
New England Journal of Medicine
Oncogene
Proc. Natl. Acad. Sci. USA
Photochemistry & Photobiology

Teaching Service

1993-Pres.	Preceptor, HHMI-NIH Research Scholars Program (5 students)
2000	Preceptor, BESIP of Whitaker Foundation of NIH
2000	Preceptor, UC Irvine Washington DC Center Program
1998-Pres.	Preceptor, NCI pre-CRTA program
1999-Pres.	Preceptor, NCI Summer Research Internship (7 students)
2000-2004	Guest Lecturer, the Topics in Molecular Epidemiology course Georgetown University Medical School

Thesis Advisor

1996	Hood College (one student, M.S. degree)
2002	Shanghai Medical University (M.D./Ph.D. program)
2004-Pres.	Fudan University (M.D./Ph.D. candidate)

Thesis Committee

1999 University of Maryland (Ph.D. degree)

Grant Support

1998-2000 Principal Investigator, Mechanism of Human liver carcinogenesis, DBS Budget Allocation, NCI, NIH

2000-Pres. Principal Investigator, Mechanism of Human liver carcinogenesis, CCR Budget Allocation, NCI, NIH

Additional Competitive Funds:

2001 Principal Investigator
SAGE transcript profiles. DBS, NCI, NIH;
Total direct costs awarded: $40,000

2001 Principal Investigator
Molecular profiling of metastatic hepatocellular carcinoma. DBS, NCI
Total direct costs awarded: $19,500

2001 Principal Investigator
Program travel. DBS, NCI, NIH
Total direct costs awarded: $15,000

2002 Principal Investigator
International Workshop. CCR, NCI, NIH,
Total direct costs awarded: $25,000

2002 Principal Investigator
International Workshop. NIH Office of Rare Diseases
Total direct costs awarded: $20,000

2003 Principal Investigator
Program travel. CCR, NCI, NIH
Total direct costs awarded: $15,000

2004 Principal Investigator
Program travel. CCR, NCI, NIH
Total direct costs awarded: $15,000

2005 Principal Investigator
Gene expression profiling of HCC by microarray
Total direct costs awarded: $60,000

2005	Principal Investigator MicroRNA expression profiling of HCC Total director costs awarded: $90,000
2006	Principal Investigator Program travel. CCR, NCI, NIH Total direct costs awarded: $15,000
2005	Principal Investigator Affymetrix HTA analysis of HCC Total director costs awarded: $50,000 (ODRS support)
2007	Principal Investigator Program travel. CCR, NCI, NIH Total direct costs awarded: $15,000

Grant Reviewer:

1998-2000	The Cancer Research Campaign
2003-Pres.	Cancer Research UK
2001	Jeffress Memorial Trust
2003-Pres.	Italian Association for Cancer Research
2004-Pres.	Research Grants Council of Hong Kong

Graduate Student Thesis Committee:

1996	Jill D. Coursen, M.S., Biomedical Science, Hood College
1999	Joo-Yeon Yoo, Ph.D., Biomedical Science, UMBC
2002	Qinghai Ye, M.D./Ph.D., Clinical Oncology, Shanghai Medical University
2006	Huliang Jia, M.D./Ph.D., Fudan University

Libin Xu, M.D./Ph.D. candidate, Molecular Immunology Branch, National Laboratory of Molecular Oncology, Cancer Institute/Hospital, Chinese Academy of Medical Sciences

Mentorship for Other Research Training Programs:

Dr. Heidi Yeh, 1994-1995, HHMI-NIH Research Scholars program
Mr. Hieu Le, 1992-1994, NCI Staying School student program
Ms. Lorraine Le, 1994-1996, NCI Staying School student program
Dr. Michael Gibson, 1995-1996, HHMI-NIH Research Scholars program
Dr. Shawn Tang, 1994-1995, NCI summer student scholar program
Dr. Nissim Khabie, 1996-1997, HHMI-NIH Research Scholars program
Dr. Anne Manicone, 1997-1998, HHMI-NIH Research Scholars program
Dr. Ann Tseng, 1998-1999, NIH pre-IRTA program
Dr. Chuan-Ging Wu, 1998-2001, NCI CRTA program

Mr. David Salvay, 2000, BESIP of Whitaker Foundation of NIH
Ms. Michele Abbasi, 2000, UC Irvine Washington DC Center Program
Ms. Alice Uy, 2000, 2001, NCI summer student scholar program
Ms. Shabina Siddique, 2000, 2001, NCI summer student scholar program
Ms. Jessica Simes, 2001-2002, NCI pre-CRTA program
Dr. Jin Woo Kim, 2001-2004, NCI CRTA program
Ms. Lavanya Viswanathan, 2002, NCI summer student scholar program
Dr. Anuradha Budhu, 2003-present, NCI CRTA program
Mr. Brian Zipser, 2003-2004, HHMI-NIH Research Scholars program
Dr. Wei Wang, 2003-2005, NCI Visiting Fellow
Dr. Vivian Takafuji, 2004-2006, NCI CRTA program
Dr. Taro Yamashita, 2005-present, NCI Visiting Fellow
Dr. Mi Jung Lim, 2005-2006, NCI Visiting Fellow
Mr. Nicholas Younes, 2005, NCI summer student scholar program
Ms. Mindy Wei, 2005, NCI summer student scholar program
Mr. Andy Chen, 2006, NCI summer student scholar program
Ms. Bhumi Patel, 2006, NCI summer student scholar program
Ms. Giang Nguyen, 2006-present, HHMI-NIH Research Scholars program
Dr. Junfang Ji, 2006-present, NCI Visiting Fellow
Dr. Stephanie Roessler, 2006-present, NCI Visiting Fellow

Research Interests:

1. Roles of oncogenes and tumor suppressor genes in regulation of genomic stability, including multiple cell cycle checkpoint controls, DNA repair and programmed cell death (apoptosis).
2. Mechanisms of liver cancer genetics.

Inventions and Patents Assigned to U.S. Government:

1. U.S. Patent No. 5,985,829. Patent Serial No. 08/675,631, filed 6/30/1996 entitled "Screening assays for compounds that cause apoptosis and related compounds" by Xin Wei Wang, Curtis Harris and Jan Hoeijmakers.
2. U.S. Patent No. 6,613,318. Patent Serial No. 60/126,069, E-232-98/0, filed 3/25/1999 entitled "Methods for identifying modulators of Gadd45 polypeptide activity, and inhibitors of such activity" by Xin Wei Wang, Curtis Harris, Jill Coursen, Albert Fornace, Qimin Zhan.
3. U.S. Patent No. 60,370,895. Patent Serial No. 09/534,811, filed 3/24/2000 entitled "Methods for Identifying Inhibitors of GADD45 Polypeptide Activity, and Inhibitors of Such Activity" by Xin Wei Wang, Curtis C. Harris, Albert J. Fornace, Jr., Jill D. Coursen, Qimin Zhan.
4. U.S. PHS Employee Invention. The Establishment of telomerase-immortalized human liver epithelial cell lines.
5. U.S. PHS Employee Invention (2002). Methods of diagnosing potential for developing hepatocellular carcinoma or metastasis and of identifying therapeutic targets.

6. U.S. Patent application (2005). Molecular diagnosis of liver cancer metastasis and recurrence by signatures of non-cancerous immune cells and its therapeutic utility by modulating the MU-Th1/MS-Th2 cytokine profile through targeting CSF-1 or OPN.
7. U.S. Patent application (2005). Compositions and Methods for Diagnosis and Treatment of Metastatic Disease.
8. U.S. Patent application (2005). Method of Screening for hepatocellular carcinoma.
9. U.S. PHS Employee Invention (2006). A novel diagnostic and therapeutic strategy for metastatic hepatocellular carcinoma by targeting a unique region of osteopontin.
10. U.S. Patent application (2006). Methods of determining the prognosis of an adenocarcinoma.
11. U.S. PHS Employee Invention (2006). A unique microRNA expression signature predicts survival, metastases and recurrence in hepatocellular carcinoma.
12. U.S. Patent No. 7,125,850B2. Patent Serial No. 11/297,160, filed 12/7/2005 entitled "Methods for Identifying Inhibitors of GADD45 Polypeptide Activity, and Inhibitors of Such Activity".
13. International Patent Application No. PCT/US06/42591. Claims Priority U.S. Provisional Patent Application No. 60/732,332 filed 11/1/2005 entitled "Method of Screening for Hepatocellular Carcinoma".

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BIBLIOGRAPHY

Articles in Journals

1. Huang, C. C., Han, J. X., Yue, X. F., Shen, Z. M., Wang, X. W., Wu, F. G. and Xu, B.: Cytotoxicity and sister chromatid exchanges induced *in vitro* by six anticancer drugs developed in the People's Republic of China. Journal of National Cancer Institute 71: 841-847, 1983.

2. Wang, X. W., Yue, X. F., Han, J. X., Wu, F. G., Shen, Z. M., Xu, B. and Huang, C. C.: Studies on cytotoxicity and induction of sister chromatid exchanges in V79 cells with three antitumor agents. KEXUE TONGBAO 29: 1268-1271, 1984.

3. Wang, X. W., Shen, Z. M., Yang, J. L. and Xu, B.: Inhibitory effect of hydroxy-camptothecin on colony formation of KB cells and DNA damage. Acta of Pharmaceutics Sinica 21: 492-497, 1986.

4. Sugiyama, M., Wang, X. W. and Costa, M.: Comparison of DNA lesions and cytotoxicity induced by calcium chromate in human, mouse and hamster cell lines. Cancer Res. 46: 4547-4551, 1986.

5. Wang, X. W., Yu, W. J., Shen, Z. M., Yang, J. L. and Xu, B.: Cytotoxicity of hydroxycamptothecin and four other antineoplastic agents on KB cells. Acta of Pharmacology Sinica 8: 86-90, 1987.

6. Conway, K., Wang, X. W., Xu, L. and Costa, M.: Effect of magnesium on nickel-induced genotoxicity and cell transformation. Carcinogenesis 8: 1115-1121, 1987

7. Zelikoff, J. T., Li, J. H., Hartwig, A., Wang, X. W., Costa, M. and Rossman, T. G.: Genetic toxicology of lead compounds. Carcinogenesis 9: 1727-1732, 1988.

8 Wang, X. W., Imbra, R. J. and Costa, M.: Characterization of mouse cell lines resistant to nickel (II) ions. Cancer Res. 48: 6850-6854, 1988.

9. Imbra, R. J., Wang, X. W. and Costa, M.: Characterization of a nickel resistant mouse cell line. Biological Trace Element Research 21: 97-103, 1989.

10. Wang, X. W. and Çosta, M.: Alteration of nickel binding proteins in nickel resistant cell. Cancer Comm. 1: 351-358, 1989.

11. Klein, C. B., Conway, K., Wang, X. W., Bhamra, R. F., Lin, X. H., Cohen, M. D., Lois, A., Barrett, J. C. and Costa, M.: Senescence of nickel-transformed cells by a mammalian X chromosome: possible epigenetic control. Science 251: 796-799, 1991.

12. Wang, X. W. and Costa, M.: Changes in protein phosphorylation in wild type and nickel-resistant cells and their involvement in morphological elongation. Biol. Metal 4: 201-206, 1991.

13. Wang, X. W., Lin, X. H., Klein, C. B., Bhamra, R. F., Lee, Y-W. and Costa, M.: A conserved region in human and Chinese hamster X chromosomes can induce cellular senescence of nickel-transformed Chinese hamster cell lines. Carcinogenesis 13: 555-561, 1992.

14. Wang, X. W., Forrester, K., Yeh, H., Feitelson, M. A., Gu, J-R. and Harris, C. C.: Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proc. Natl. Acad. Sci. USA 91: 2230-2234, 1994.

15. Wang, X. W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J.-M., Wang, Z., Friedberg, E. C., Evans, M. K., Taffe, B. G., Bohr, V. A., Weeda, G., Hoeijmakers, J. H. J., Forrester, K. and Harris, C. C.: p53 modulation of TFIIH associated nucleotide excision repair activity. Nature Genetics 10: 188-195, 1995.

16. Forrester, K., Lupold, S. E., Ott, V. L., Chay, C. H., Wang, X. W. and Harris, C. C.: Effects of p53 mutants on wild-type p53-mediated transactivation are cell type dependent. Oncogene 10: 2103-2111, 1995.

17. Wang, X. W., Gibson, M., Vermulen, W., Yeh, H., Forrester, K., Sturzbacher, H.-W., Hoeijmakers, J. H. J. and Harris, C. C.: Abrogation of p53-induced apoptosis by the hepatitis B viral X gene. Cancer Res. 55: 6012-6016, 1995.

18. Forrester, K., Ambs, S., Lupold, S. E., Kapust, R. B., Spillare, E. A., Weinberg, W.C., Felley-Bosco, E., Wang, X. W., Geller, D. A., Billiar, T. R. and Harris, C. C.: Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase (NOS2) expression by wild-type p53. Proc. Natl. Acad. Sci. USA 93: 2442-2447, 1996.

19. Wang, X. W., Vermulen, W., Coursen, J. D., Gibson, M. J., Lupold, S. E., Forrester, K., Xu, G., Elmore, L., Yeh, H., Hoeijmakers, J. H. J. and Harris, C. C.: The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. Genes & Development 10: 1219-1232, 1996.

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Control of apoptosis by p53

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The p53 tumor suppressor acts to integrate multiple stress signals into a series of diverse antiproliferative responses. One of the most important p53 functions is its ability to activate apoptosis, and disruption of this process can promote tumor progression and chemoresistance. p53 apparently promotes apoptosis through transcription-dependent and -independent mechanisms that act in concert to ensure that the cell death program proceeds efficiently. Moreover, the apoptotic activity of p53 is tightly controlled, and is influenced by a series of quantitative and qualitative events that influence the outcome of p53 activation. Interestingly, other p53 family members can also promote apoptosis, either in parallel or in concert with p53. Although incomplete, our current understanding of p53 illustrates how apoptosis can be integrated into a larger tumor suppressor network controlled by different signals, environmental factors, and cell type. Understanding this network in more detail will provide insights into cancer and other diseases, and will identify strategies to improve their therapeutic treatment.
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Tumor suppressors act to maintain tissue homeostasis, that is, to control the number and behavior of cells in a particular tissue within an organism (Hussain and Harris, 1998). To do so, they typically regulate one or more processes that prevent aberrant proliferation (Vogelstein *et al.*, 2000; Vousden and Lu, 2002). p53 is the most extensively studied tumor suppressor, and acts in response to diverse forms of cellular stress to mediate a variety of antiproliferative processes. Hence, p53 can be activated by DNA damage, hypoxia, or aberrant oncogene expression to promote cell-cycle checkpoints, DNA repair, cellular senescence, and apoptosis. As a consequence, disruption of p53 function promotes checkpoint defects, cellular immortalization, genomic instability, and inappropriate survival, allowing the continued proliferation and evolution of damaged cells. Given the profound proliferative advantage produced by loss of p53 function, it is not surprising that *p53* is the most commonly inactivated tumor suppressor gene in human cancer (Hussain and Harris, 1998; Beroud and Soussi, 2003).

Although most of the attention on p53 has focused on its role in cancer, chronic activation of this key biological pathway may be equally as deleterious as its inactivation. In fact, hyperactivation of p53 has been associated with a variety of degenerative diseases such as arthritis, multiple sclerosis (Wosik *et al.*, 2003), and neuropathies (Mattson *et al.*, 2001), as well as with the exacerbation of ischemic damage from strokes or cardiac arrest (Komarova and Gudkov, 2001). Moreover, studies using mouse models suggest that acute p53 activation contributes to the side effects of cancer chemotherapy, whereas chronic p53 activation can contribute to aging (Komarova and Gudkov, 2001; de Stanchina and Lowe, 2002; Tyner *et al.*, 2002). Together, these observations imply that p53 activity must be a tightly regulated, with too much, or too little p53 producing, or contributing to, disease.

One of the most extensively studied areas in p53 research surrounds its ability to control apoptosis. The first hint that p53 could control apoptosis came from work by Oren and co-workers who reintroduced *p53* into a *p53*-deficient myeloid leukemia cell line (Yonish-Rouach *et al.*, 1991). Here, p53 induced apoptosis in a manner that could be countered by a prosurvival cytokine. Subsequently, evidence that endogenous p53 could control apoptosis was obtained from studies using thymocytes from *p53* knockout mice, which showed that p53 was required for radiation-induced apoptosis, but not cell death induced by several other stimuli (Clarke *et al.*, 1993; Lowe *et al.*, 1993b). These studies, together with the observation that loss of apoptosis correlated with tumor progression in *p53*-null transgenic mice (Symonds *et al.*, 1994; Parant and Lozano, 2003), implied that apoptosis contributes to p53's tumor suppressor activity. Furthermore, the fundamental role apoptosis plays in the biology of p53 is emphasized by its evolutionary conservation in both *Drosophila* (Brodsky *et al.*, 2000; Jin *et al.*, 2000; Ollmann *et al.*, 2000) and *C. elegans* (Frantz, 2001; Schumacher *et al.*, 2001), where the respective orthologs are an important component of damage surveillance.

In addition to its role in suppressing tumorigenesis, p53-dependent apoptosis contributes to chemotherapy-induced cell death (see, for review, Johnstone *et al.*, 2002). This was first demonstrated in studies using oncogenically transformed cells treated *in vitro* and *in*

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vivo (Lowe *et al.*, 1993a), and was subsequently extended to a variety of settings. Consistent with the potential role for p53 in modulating chemotherapy in human cancers, loss of p53 function was linked to chemoresistance in certain tumor types (Wallace-Brodeur and Lowe, 1999; Johnstone *et al.*, 2002). Although the overall contribution of p53 to chemosensitivity in human patients remains under debate, these studies revealed the potential importance of apoptosis in cancer chemotherapy and initiated a link between cancer development and therapy. Thus, a more complete understanding of the p53 apoptotic program presents hope for improved assays for cancer diagnosis and prognosis, and may suggest rational strategies to improve therapy. Here, we summarize the roles, regulation, and execution of the p53 apoptotic program.

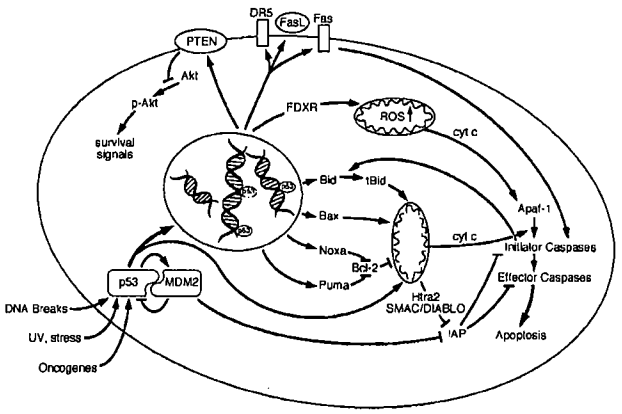

Figure 1 A model for p53-induced apoptosis by simultaneous targeting of distinct points in the apoptotic network.

The apoptotic program

Apoptosis is a complex process that proceeds through at least two main pathways (extrinsic and intrinsic), each of which can be regulated at multiple levels. The extrinsic pathway, which consists of cell surface receptors, their inhibitory counterparts ('decoy death receptors'), and their associated cytoplasmic proteins, can be modulated by altering the number of each type of receptor, thus setting the rheostat that determines the sensitivity of cells to various ligands (Peter and Krammer, 2003). Additional points of regulation include the expression levels of these activating ligands and the cytoplasmic adapter molecules (e.g. FADD) required for procaspase activation upon ligand binding, as well as the death inhibitory molecules (e.g. FLIP) (Peter and Krammer, 2003).

The intrinsic pathway centers on the mitochondria, which contain key apoptogenic factors such as cytochrome *c*, AIF, SMAC/DIABLO, Htra2/Omi (see, for review, Kroemer and Reed, 2000), and endoG (Li *et al.*, 2001). Major regulators of the intrinsic pathway are the pro- and antideath members of the Bcl-2 family (Tsujimoto, 2003). These proteins reside at, or translocate to the mitochondria, controlling the release of the aforementioned factors. Furthermore, the inhibitor of apoptosis proteins (IAPs) provides another level of control for both the intrinsic and extrinsic pathways, which often cooperate – depending on cell type and stimulus – to kill a cell in an orderly way. p53 serves as a regulator of the apoptotic process that can modulate key control points in both the extrinsic and intrinsic pathways (see Figure 1 for an overview).

Downstream effectors of p53 in apoptosis

p53 is a transcription factor capable of binding DNA in a sequence-specific fashion (Ko and Prives, 1996). Interestingly, virtually all tumor-derived mutants are defective in their ability to bind DNA specifically, implying that there is a strong selective pressure to disable this p53 property during the course of tumorigenesis. Accordingly, the proapoptotic activity of p53 has been linked to its transactivation capabilities through several approaches. For example, the ability of p53 to transactivate target genes has been correlated to apoptosis in some, although not all, structure function studies (Attardi *et al.*, 1996; Chen *et al.*, 1996), and p53 can directly activate the transcription of genes known to promote apoptosis (el-Deiry, 1998; Yu *et al.*, 1999; Sax and el-Deiry, 2003). Moreover, knockin mice expressing transcriptionally dead, but DNA-binding proficient p53 are defective in apoptosis (Jimenez *et al.*, 2000). This latter result provides compelling *in vivo* evidence that transactivation is essential for p53 to promote apoptosis in normal cells.

Transcriptional control of the Bcl-2 family

The most intuitive link between p53-mediated transactivation and apoptosis comes from its ability to control transcription of proapoptotic members of the Bcl-2 family. These include the 'multidomain' Bcl-2 family member *Bax* (Miyashita *et al.*, 1994), as well as the 'BH3-only' members *Puma* (Nakano and Vousden, 2001), *Noxa* (Oda *et al.*, 2000a), and *Bid* (Sax *et al.*, 2002). In all cases, the promoters of these genes harbor consensus p53 response elements that are capable of binding p53 *in vitro* and conferring p53 responsiveness to reporter genes *in vivo*. Precisely, how these proteins act downstream of p53 to mediate apoptosis is an active area of research, but their net effect is to increase the ratio of pro- to antiapoptotic Bcl-2 proteins, thereby favoring the release of apoptogenic proteins from the mitochondria, caspase activation, and apoptosis.

Gene targeting studies in both mice and cultured cells support the notion that proapoptotic members of the Bcl-2 family can act downstream of p53 during apoptosis. For instance, *Bax*-deficient mouse embryo fibroblasts (MEFs) are desensitized to oncogene-induced apoptosis (which is also p53 dependent), leading to increased transformation *in vitro* and tumorigenesis *in vivo* (McCurrach *et al.*, 1997; Yin *et al.*, 1997). Moreover, disruption of either *Bax* or *Puma* in HCT116 cells

produces various degrees of apoptotic defects (Zhang *et al.*, 2000; Yu *et al.*, 2003). Still, studies showing that the phenotypes of genetically targeted mice (or cells from such mice) are similar (e.g. *p53* null vs. *Bax* null MEFs) do not rule out the possibility that two gene products act in parallel pathways to produce a common phenotype. However, the fact that *p53* loss attenuates the expression of the downstream targets suggests that the phenotypes of the null effectors reflect defects in p53-mediated apoptosis.

Adding to the complexity of the program, the effects of gene disruption can vary depending on experimental setting. For example, *Bax*-deficient thymocytes are sensitive DNA-damaging signals, even though oncogene-transformed MEFs lacking *Bax* show apoptotic defects. Moreover, disruption of *Bax* does not accelerate Myc-induced lymphomagenesis, even though overexpression of Bcl-2 (which antagonizes Bax) readily does so (JSF and SWL, unpublished observations). These observations highlight a recurrent theme in studies on p53-dependent apoptosis – the program details are often context dependent.

Transcriptional control of the apoptotic machinery

A body of work indicates that p53-mediated apoptosis proceeds primarily through the intrinsic apoptotic program (e.g. Soengas *et al.*, 1999; Schuler *et al.*, 2000). In addition to controlling factors that act upstream of the mitochrondria, p53 can also transactivate several components of the apoptotic effector machinery. One of these components is the gene encoding Apaf-1 (Kannan *et al.*, 2001; Moroni *et al.*, 2001; Robles *et al.*, 2001), which acts as a coactivator of caspase-9 and helps initiate the caspase cascade. In addition, p53 can upregulate expression of caspase-6, often considered an effector caspase, leading to enhanced chemosensitivity of some cell types (MacLachlan *et al.*, 2002). It seems likely that this point of control is not crucial for the initiation of apoptosis, but may potentiate cell death in the presence of released cytochrome *c* (Juin *et al.*, 2002). A similar model has been proposed for E2F, which both promotes apoptosis and can increase caspase expression through a direct transcriptional mechanism (Nahle *et al.*, 2002).

Transcriptional control of the extrinsic pathway

The extrinsic apoptotic pathway is also regulated by p53, although the overall contribution of this regulation to p53-mediated cell death is poorly understood. For example, the *Fas/CD95* (Owen-Schaub *et al.*, 1995; Muller *et al.*, 1998) and *DR5* (Wu *et al.*, 1997) death receptor loci, as well as the gene encoding for Fas ligand, *TNFSF6* (Maecker *et al.*, 2000), are each direct p53 targets. Moreover, the ability of p53 to transactivate *Bid* may facilitate crosstalk between the extrinsic and intrinsic pathways (Sax *et al.*, 2002). Consequently, p53 may sensitize cells to death receptor ligands, either inducing apoptosis directly or enhancing cell death in ligand-rich environments. Interestingly, disabling this sensitization by *p53* mutation can promote drug resistance in some contexts (Fulda *et al.*, 1998; Petak *et al.*, 2000), and provide a degree of immune privilege to tumor cells (Green and Ferguson, 2001).

Other transcriptional targets

Beyond the core constituents of the intrinsic and extrinsic apoptotic pathways, p53 transcriptionally activates other genes that have been linked to apoptosis. In most instances, these genes have p53 response elements in their promoters and can modulate apoptosis when over- or underexpressed. For example, *PERP* was identified in a screen for apoptosis-specific genes regulated by p53, and is capable of inducing apoptosis in *p53*-null cells, albeit not to the same extent as p53 (Attardi *et al.*, 2000). In a similar fashion, *PIDD* (p53-induced protein with a death domain) was identified as a p53-responsive gene induced following shift of an erythroleukemia cell line, containing temperature-sensitive p53, to the permissive (wild-type p53) temperature (Lin *et al.*, 2000). Although its precise role in apoptosis remains to be determined, suppression of *PIDD* inhibits apoptosis, whereas enforced PIDD expression induces cell death. *p53DINP1* (p53-dependent damage-inducible nuclear protein 1) (Okamura *et al.*, 2001) and *p53AIP1* (p53 apoptosis-inducing protein 1) (Oda *et al.*, 2000b) are both p53-inducible genes that appear to form part of a mini network in the p53 apoptotic program. p53DINP1 interacts with a multiprotein kinase complex capable of phosphorlyating p53 (Okamura *et al.*, 2001) on serine 46, which correlates with the transcriptional activation of *p53AIP1*. p53AIP, in turn, disrupts mitochondrial function and is sufficient to induce cell death in a number of tumor cell lines when overexpressed (Oda *et al.*, 2000b; Matsuda *et al.*, 2002).

p53 targets survival signaling

In addition to its ability to transactivate genes that directly promote apoptosis, p53 can also induce genes that short-circuit antiapoptotic pathways. The most obvious example of this is the ability of p53 to regulate PTEN, a negative regulator of the PI3 kinase pathway. The PI3 kinase pathway translates signals from receptor tyrosine kinases to changes in cellular physiology. Prosurvival cytokines lead to the activation of PI3 kinase, the production of phosphotidyl inositol-3,4-P_2 and -3,4,5-P_3, and activation of downstream effectors, including Akt/PKB. Akt, in turn, phosphorylates effector molecules that can regulate survival in several ways (Vivanco and Sawyers, 2002). PTEN is a lipid phosphatase that attenuates PI3 kinase signaling by dephosphorylating 3′-phosphorylated phosphatidylinositides. Notably, p53 can transactivate the *PTEN* promoter leading to increases in PTEN expression, although the induction is relatively modest. Nevertheless, these changes can have profound effects, as disruption of *PTEN* can compromise p53-mediated apoptosis in some cell types (Stambolic *et al.*, 2001). Thus, p53 can counteract survival signals from the

microenvironment, presumably reducing the threshold needed for proapoptotic factors to trigger cell death.

Redox metabolism

Induction of p53 has also been shown to produce changes in REDOX metabolism, leading to increases in reactive oxygen species (ROS) prior to the onset of apoptotic cell death (Polyak *et al.*, 1997; Hwang *et al.*, 2001). Although precisely how this regulates cell death is poorly understood, p53 can upregulate a number of genes that affect REDOX metabolism and certain antioxidants can suppress p53-mediated cell death (Polyak *et al.*, 1997; Hwang *et al.*, 2001). One gene, *ferredoxin reductase* (*FDXR*), is specifically upregulated after treatment of colon carcinoma cells with the chemotherapeutic agent 5-fluorouracil (5-FU) only in cells containing p53 (Hwang *et al.*, 2001). Interestingly, disruption of *FDXR* decreases the amount of ROS and reduces apoptosis following 5-FU treatment. Furthermore, the cellular REDOX state was shown to impact the levels and activity of p53 as the two studies have implicated oxidoreductases in the regulation of p53 stability (Asher *et al.*, 2001; Chang *et al.*, 2003). Thus, the activation of p53 leads to an increase in ROS that, perhaps by interfering with mitochondrial function and/or integrity, contributes to cell death. In addition, the higher levels of ROS appear to be part of feedforward loop that stabilizes p53 resulting in more p53 activity.

P53-mediated transrepression

While most studies investigating the action of p53 in apoptosis have focused on its transactivation functions, p53 also has transrepression capabilities that may contribute to apoptosis (Mack *et al.*, 1993; Zhang *et al.*, 1998, 1999). How p53 represses transcription is not fully established, but appears to involve its ability to recruit histone deacetylases to certain genes through the mSin3a corepressor (Murphy *et al.*, 1999). One of the targets of p53-mediated repression is *Survivin*, which encodes an IAP capable of inhibiting apoptosis when overexpressed (Ambrosini *et al.*, 1997). In principle, *p53* mutations might contribute to the high frequency of Survivin overexpression observed in human tumors (Hoffman *et al.*, 2002). Nevertheless, Survivin also has important roles in mitosis, and as such, the extent to which dysregulation of Survivin's antiapoptotic activity contributes to tumor progression is unclear. However, at least under certain circumstances, such as hypoxia, the ability of p53 to transrepress may be more important for inducing apoptosis than its transactivation function (Koumenis *et al.*, 2001). How this translates to p53's role in tumor suppression is unclear.

Nontranscriptional modes of action

Although p53 can up- and downregulate gene transcription, its influence on apoptosis may not end there. Indeed, p53 may also control apoptosis through transcription-independent mechanisms (Caelles *et al.*, 1994; Wagner *et al.*, 1994; Haupt *et al.*, 1995; Chen *et al.*, 1996; Kokontis *et al.*, 2001; Dumont *et al.*, 2003). For the most part, studies linking apoptosis to transcription-independent functions of p53 involve overexpression of mutant p53 proteins at unphysiologic levels, and so the contribution of this mode of regulation to apoptosis induced by endogenous p53 is not well established. However, recent studies suggest that stress-induced accumulation of p53 can occur in the mitochondria (Mihara *et al.*, 2003). Here, mitochondrial redistribution of p53 precedes cytochrome *c* release and caspase activation, and occurs only during p53-dependent cell death. This mitochondrial p53 appears to be proapoptotic, since direct targeting of p53 to mitochondria can promote apoptosis in *p53*-deficient cells (Mihara *et al.*, 2003). Moreover, polymorphic p53 variants that have different apoptotic potential show a differential ability to localize to the mitochondria, with the least proapoptotic being deficient in this property (Dumont *et al.*, 2003).

Although the transcription-independent functions of p53 are intriguing, they are unlikely to be essential for p53-mediated apoptosis. As indicated earlier, sequence-specific DNA binding appears to be the primary p53 function selected against during tumorigenesis, and normal cells harboring an endogenous p53 mutant that is defective in transactivation capabilities do not undergo apoptosis (see above). Although some tumor-derived mutants may also be defective in their ability to act at the mitochondria, the preponderance of evidence suggests that p53's role in transcriptional activation is the crucial activity in regulating apoptosis. It seems more likely that the nontranscriptional activities of p53 play an auxiliary role, potentiating p53-mediated cell death.

Coordination of the apoptotic program by p53

Why would a transcription factor evolve to use so many distinct mechanisms to produce the same biological end point? Such a scenario seems extremely inefficient and redundant for a protein that appears dispensable for normal development (Donehower *et al.*, 1992). However, this paradox can be reconciled if one views the various p53 targeting mechanisms not as isolated circuits, but as part of a coordinated process that targets key nodes of the apoptotic network. By simultaneously targeting several levels of the apoptotic program, p53 increases the probability that the process goes forward and ensures a well-coordinated program once the process is initiated. Moreover, such a program builds in a variety of control points that integrate many elements of the cellular milieu.

Notably, the fact that p53 simultaneously targets multiple 'death' circuits to coordinate an apoptotic response explains, in part, why no single p53 effector molecule can account for all of p53's proapoptotic activity. Moreover, since each circuit functions as part of a larger network rather than a specific linear pathway,

they can be affected in different ways by the cell type, microenvironment, apoptotic stimulus, or genetic background. As a consequence, one or more circuits may stand out as the crucial element in a particular cell type, or at different stages during tumor progression.

Such a model may explain some of the apparently conflicting data in the literature. For example, disruption of *Bax* compromises p53-mediated apoptosis in oncogenically transformed fibroblasts (McCurrach *et al.*, 1997) and developing tumors of the choroid plexus (Yin *et al.*, 1997), but has no obvious effect on p53-mediated apoptosis in normal thymocytes (Knudson *et al.*, 1995). Perhaps, cell type differences or changes in the apoptotic network produced by oncogene expression alter the relative importance of the Bax-regulated circuit for p53-mediated cell death. Moreover, disruption of the p53 effector *Apaf-1* attenuates p53-mediated apoptosis in transformed fibroblasts (Soengas *et al.*, 1999), melanoma cells (Soengas *et al.*, 2001), and the developing central nervous system and lens of the *Rb*-deficient mouse (Guo *et al.*, 2001). Conversely, thymocytes lacking *Apaf-1* respond normally to irradiation (Marsden *et al.*, 2002). In most instances, however, caspase-9 is activated during apoptotic cell death in the Apaf-1-expressing counterparts, implying that the p53-mediated programs are not fundamentally different, but that the "Critical Nodes" may vary. Here again, it is possible that aberrantly proliferating cells may rewire their apoptotic networks, leading to a greater relative reliance on the apoptosome for efficient cell death. Alternatively, thymocytes and cells of the hematopoietic compartment are hardwired to die in response to many stimuli, and may have in place more redundant or efficient death effector mechanisms. In other words, in the absence of *Apaf-1*, some cell types bypass their 'first choice' pathway and use alternative methods to activate effector caspases and induce apoptosis. Clarifying these complexities represents a challenge, but also offers hope for more selective intervention of the p53-apoptotic program.

Regulation of p53-dependent apoptosis: deciding cell fate

In addition to its ability to promote apoptosis, p53 can also induce cell cycle arrest, cellular senescence, and directly influence DNA repair. What determines whether p53 induces apoptosis rather than another outcome? Initial studies suggested that the most important determinant of this decision is cell type or tissue of origin. For example, γ-irradiation of fibroblasts engages a p53-dependent G1 cell cycle arrest, while in thymocytes, it produces a p53-mediated apoptosis (Kuerbitz *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993b). However, cell type differences alone cannot explain the different outcomes, since fibroblasts expressing the E1A or Myc oncoproteins undergo p53-dependent apoptosis in response to γ-irradiation or other forms of DNA damage. Similarly, lymphoma cells typically undergo a p53-dependent apoptotic program in response to the chemotherapeutic drug cyclophosphamide; however, if these same lymphoma cells overexpress Bcl-2 (which prevents apoptosis), the cells undergo a p53-dependent program of cellular senescence (Schmitt *et al.*, 2002b). Finally, enforced expression of p53 promotes apoptosis in myeloid leukemia cells in a manner that is suppressed in the presence of IL-6, despite the same levels of p53 expression (Yonish-Rouach *et al.*, 1991). Therefore, both genetic background and microenvironment significantly impact p53 responses.

The outcome of p53 activation may also be influenced by the strength or nature of the p53-activating stimulus. For example, in MEFs, E1A activates p53 to promote apoptosis, whereas oncogenic Ras activates p53 to promote senescence (Lowe and Ruley, 1993; Serrano *et al.*, 1997). Interestingly, in both instances, oncogene signaling to p53 requires the ARF tumor suppressor, which stabilizes p53 by interfering with its negative regulator Mdm2 (de Stanchina *et al.*, 1998; Paramio *et al.*, 2001). Although the molecular basis for these differences remains to be established, microarray studies show that distinct p53 activating stimuli (e.g. γ-radiation *vs* UV) can produce unique p53-dependent gene expression patterns. Thus, the upstream signal can impact the downstream response (Zhao *et al.*, 2000).

Quantity vs *quality*

How might p53 interpret contextual factors and respond accordingly? One model assumes that different p53 outcomes are sensitive to the magnitude or robustness of the p53 response. In principle, the amplitude or duration of the activating signal, or a variety of factors that affect other signaling pathways in the cell, may enhance or suppress p53 activation to impact the p53 response. For example, Ras signaling can induce Mdm2 in a p53-dependent manner, thereby blunting p53 activation in response to DNA damage in Ras-expressing cells, compared to non-Ras-expressing cells (Ries *et al.*, 2000). Notably, the quantitative model assumes the existence of p53-responsive genes containing promoter elements with differing binding affinities, or perhaps the engagement of nontranscriptional p53 activities dependent on p53 dose. In the case of transcriptional targets, a subset of promoters should be activated only when the expression level of p53 reaches a certain threshold. Activation of this subset of promoters would lead to unique transcription profiles altering the cellular response to p53. Such a model is consistent with studies using conditional p53 expression systems, where low p53 levels promote arrest and higher p53 levels promote apoptosis (Chen *et al.*, 1996; Zhao *et al.*, 2000). Nevertheless, the underlying mechanism for these effects, and whether they relate to differential affinity for certain p53-responsive promoters, remains to be determined.

It is also possible that nonquantitative (i.e. qualitative) mechanisms can influence the outcome of p53

activation. In one scenario, the downstream consequences of p53 activation are the same irrespective of biological outcome, but contextual factors ('collateral signals') influence how the cell interprets the signal. Collateral signals may differ depending on the cell or tissue type, the genetic background, or the status of other signaling pathways in the cell. As one example, enforced expression of Myc can shift the outcome of p53 activation from cell-cycle arrest to apoptosis. The underlying mechanism for this effect appears to depend on the ability of DNA damage to induce p21, the cyclin-dependent kinase inhibitor linked to p53-mediated arrest (Seoane *et al.*, 2002). In cells overexpressing Myc, p53 is unable to transactivate *p21* because Miz1 (an Myc relative and binding partner) recruits Myc to the *p21* promoter, where the complex prevents p53-mediated transcription. Interestingly, Myc does not interfere with p53-mediated transcription of key apoptosis mediators and, as such, acts as a 'collateral signal' that makes apoptosis the dominant pathway upon DNA-damaging treatment.

In another scenario, p53 itself is fundamentally different depending on the activating stimulus and/or cell type, leading to qualitative differences in signal output. Such a model is consistent with the observation that gamma or UV radiation can induce different p53 target genes in the same cell type (Zhao *et al.*, 2000). Interestingly, these distinct stimuli lead to different post-translational modifications on p53 (Kapoor and Lozano, 1998; Lu *et al.*, 1998; Webley *et al.*, 2000). In principle, p53 molecules with distinct modifications may have different promoter preferences or recruit distinct transcriptional coactivators, thus leading to the activation of a distinct population of p53 target genes and different cellular responses. In this regard, DNA damage and hypoxia produce different p53 modifications, which correlate with the ability of p53 to associate with different transcriptional coactivators and repressors (Koumenis *et al.*, 2001). Furthermore, p53 modifications can influence the ability of p53 to bind its negative regulator Mdm2 (a p53-inducible E3 ubiquitin ligase capable of targeting p53 for degradation), resulting in a higher level of p53 protein and signaling (Shieh *et al.*, 1997). In other words, a qualitative effect on p53 (phosphorylation) has a quantitative effect on p53 signaling.

Which post modifications might influence p53 activity?

The most well-studied p53 modification is phosphorylation, and indeed p53 can be phosphorylated on many residues (see, for review, Meek, 1999; Prives and Hall, 1999). Determining whether and how specific p53 modifications are important for distinct p53 responses represents a challenge that has been difficult to address by routine structure function analysis. Currently, only phosphorylation on serine 46 has been linked to the ability of p53 to promote apoptosis, where it has been shown that this form of p53 preferentially activates apoptotic effectors such as p53AIP1 (Oda *et al.*, 2000b). Nevertheless, serine 46 is not conserved in murine p53, yet murine cells are perfectly able to undergo apoptosis. Hence, other modifications and/or mechanisms must also be important. In addition, p53 can also be post-transcriptionally modified by acetylation and sumolation of certain lysine residues, and these changes may contribute to p53 activation (see, for review, Meek, 1999; Prives and Manley, 2001; Alarcon-Vargas and Ronai, 2002). Whether these modifications qualitatively influence the outcome of p53 activation remains unclear.

Whether or not p53 activation connects to the apoptotic network has important ramifications for treating cancer, and perhaps other diseases as well. For example, as discussed previously, different cell types have different default programs following p53 activation. In principle, this may influence the utility of certain chemotherapeutic agents, as many of their dose-limiting side effects arise in tissues having apoptosis as their default p53 response (e.g. intestinal epithelium and the hematopoietic system Gudkov and Komarova, 2003). Indeed, efforts are underway to increase the therapeutic window of chemotherapeutics used to treat *p53*-deficient tumor cells by inhibiting the toxic effects to such normal tissues. Conversely, the ability of oncogenes such as Myc to sensitize cells to chemotherapy may explain, in part, the therapeutic index of certain chemotherapeutic agents to begin with (Lowe and Lin, 2000; Pelengaris *et al.*, 2002). Finally, disruption of apoptosis downstream of p53 can reveal p53-dependent growth arrest programs in tumor cells that are not as effective as apoptosis at prolonging overall survival in mice treated with chemotherapy (Schmitt *et al.*, 2002b). Clearly, restoring the apoptotic programs to these tumor cells would have a therapeutic benefit.

The extended p53 family

Although p53 was an orphan for many years, it is now known to be part of a larger gene family. p73 was discovered in 1997 and shortly thereafter p63 was identified (see, for review, Yang *et al.*, 2002). Although both p63 and p73 share key functional domains with p53, including its N-terminal transactivation domain, C-terminal oligomerization domain, and a conserved DNA-binding domain, their gene organization and developmental roles are considerably more complex (Yang *et al.*, 2002). For example, in contrast to *p53*, the *p63* and *p73* genes encode for several isoforms, including variants that lack the N-terminal transactivation domain that can function as dominant negatives when overexpressed (Moll *et al.*, 2001). Moreover, studies using knockout mice reveal that p63 is required for normal epithelial stem cell function and for the proper development of several tissues (Mills *et al.*, 1999; Yang *et al.*, 1999), whereas p73 functions primarily in the central nervous system (survival, neurogenesis, and spinal fluid homeostasis) (Yang *et al.*, 2002). This contrasts with p53, which has no overt role in normal development (Donehower *et al.*, 1992).

Interestingly, both p63 and p73 have been linked to apoptosis, raising the possibility that they, like p53, may be tumor suppressors. However, the precise role of these genes in cancer development is unclear, in part, because inactivating mutations in tumors have not been identified (Yang *et al.*, 2002) and *p73*-deficient mice are not tumor prone (Yang *et al.*, 2000) (note that the early death of *p63*-null mice has precluded tumorigenicity studies to date). Nevertheless, recent studies suggest that p63 and p73 can modify apoptosis and perhaps tumor behavior. For example, overexpression of p63 and p73 induces apoptosis and upregulates p53 target genes in several cell types (Moll *et al.*, 2001). Although it is possible that this activity merely reflects the ability of either protein to take on p53 functions when sufficiently overexpressed, emerging evidence suggests that p63 and p73 can be induced in response to certain apoptotic triggers, such as DNA damage, overexpression of E2F1 or activated oncogenes (Katoh *et al.*, 2000; Soengas and Lowe, 2000). Furthermore, the transactivation domain-deficient isoforms of p63 and p73 are overexpressed in some human tumors (see, for review, Moll *et al.*, 2001; Melino *et al.*, 2002; Benard *et al.*, 2003), where they may act as dominant negatives or interfere with normal p53 function by forming mixed complexes with p53 (Moll *et al.*, 2001). Conversely, some missense p53 mutants bind p73 and interfere with chemotherapy-induced apoptosis (Bergamaschi *et al.*, 2003; Irwin *et al.*, 2003). These results may explain the gain-of-function activities of some p53 mutants and identify a potentially important mechanism of chemoresistance.

p63 and p73 may induce apoptosis through several mechanisms. In some settings, p63 and/or p73 may act independently of p53 to promote cell death. In principle, such programs may induce a program mechanistically similar to p53-mediated apoptosis under different circumstances or settings than p53, perhaps in developmental settings or compensatory circumstances where p53 is not expressed. Alternatively, p63 and/or p73 may act in parallel with p53 to promote apoptosis. For example, E2F-1 (which activates p53 to promote apoptosis) can induce p73, leading to apoptosis in *p53*-null cells (Irwin *et al.*, 2000; Lissy *et al.*, 2000; Stiewe and Putzer, 2000). Such a cooperative mechanism might explain the ability of many p53 activating stimuli to promote apoptosis in *p53*-deficient cells, albeit to a lesser extent than if p53 is present.

In some settings, p63 and p73 may be part of the central mechanism whereby p53 promotes apoptosis. Consistent with this possibility, oncogene-expressing fibroblasts and embryos from double mutant mice lacking both *p63* and *p73* are as resistant to DNA damage-induced cell death as those from animals lacking *p53* (Flores *et al.*, 2002). However, double mutant cells still induce p53 in response to stress, leading to the activation of some target genes. Yet, these cells do not recruit p53 to apoptosis-specific promoters and are unable to activate p53-responsive genes linked to apoptosis (Flores *et al.*, 2002). Nevertheless, while these results are provocative, the true impact of p63 and p73 on p53-dependent apoptosis in human tumors remains to be established.

Is apoptosis important for tumor suppression by p53?

The p53 tumor suppressor was initially identified as the 'guardian of the genome' based on its ability to mediate a G1 arrest following DNA damage (Kuerbitz *et al.*, 1992; Lane, 1992). However, as indicated above, p53 is now known to act in many cellular processes, including cell-cycle checkpoints, DNA repair, senescence, angiogenesis, surveillance of genomic integrity, and apoptosis (Ko and Prives, 1996; Evan and Vousden, 2001). In principle, disruption of any one or combination of these may produce an advantage during tumor development and indeed, it is widely assumed that the high frequency of *p53* mutations in human tumors reflects the profound advantage a developing tumor cell receives by simultaneous loss of all p53 functions (Vogelstein *et al.*, 2000).

How do we know that apoptosis is important for p53's tumor suppressor activity? Other than intuition, the importance of apoptosis for p53-mediated tumor suppression is inferred from correlative studies linking *p53* loss to apoptotic defects during the progression of murine and human tumors (Bardeesy *et al.*, 1995; Attardi and Jacks, 1999), as well as by functional studies demonstrating that strictly antiapoptotic activities can accelerate tumorigenesis in transgenic mice (Strasser *et al.*, 1990; Yin *et al.*, 1997; Eischen *et al.*, 2001). Furthermore, certain *p53* wild-type tumors harbor mutations that can suppress apoptosis downstream of p53 (Meijerink *et al.*, 1998; Ionov *et al.*, 2000; Soengas *et al.*, 2001), and some tumor-derived p53 mutants are defective at inducing apoptosis but not cell-cycle arrest (Aurelio *et al.*, 2000). Nevertheless, because of the many other defects present in *p53* mutant tumor cells, it has been difficult to assess the overall contribution of apoptosis to p53-mediated tumor suppression.

Attempts to address this issue directly have used mouse models to determine whether disruption of individual p53 effectors can recapitulate the effects of p53 inactivation during tumorigenesis. Although inactivation of a single p53 effector has not been able to phenocopy *p53* loss, it has been difficult to determine whether this observation reflects the requirement of multiple p53 effectors for apoptosis or the contribution of other p53 effector programs. This caveat has recently been addressed in Myc-induced lymphomas arising in Eμ-*myc* transgenic mice (Schmitt *et al.*, 2002a). Here, the effects of *p53* deficiency on lymphomagenesis were compared to the effects of Bcl-2 expression – a potent antiapoptotic gene that acts downstream of p53 to ablate p53-mediated cell death completely. Interestingly, lymphomas arising in the presence of Bcl-2 arose with the same accelerated onset as *p53*-null lymphomas and displayed a similar disseminated pathology. Moreover, Bcl-2 overexpression prevented *p53* mutations in mice heterozygous for *p53*, indicating that disruption of apoptosis downstream of p53 could compensate for *p53* loss in this model. Interestingly, whereas *p53*-null

lymphoma cells had cell-cycle checkpoint defects and were highly aneuploidy, Bcl-2-expressing lymphomas (harboring intact *p53*) retained these checkpoints and were largely diploid. Thus, in this system, apoptosis is the only p53 function selected against during lymphomagenesis, whereas the cell cycle checkpoint defects and genomic instability are by-products of *p53* loss. Importantly, these experiments argue that not all p53 functions contribute to tumorigenesis.

While the Eμ-*myc* model represents a situation in which apoptosis is essential for p53-mediated tumor suppression, this is not to say that apoptosis is the only function of p53 that is important. In fact, there appears to be two variables that determine which p53 functions contribute to its tumor suppressor activities – context and evolution. Context – the cell type or initiating oncogenic event – can dramatically influence the response of p53 and, hence, what is the primary tumor suppressor function of p53 that must be overcome for tumor expansion. For example, the c-Myc oncoprotein drives proliferation, but also promotes apoptosis. Thus, in situations where Myc activation is the initiating oncogenic event, such as the Eμ-*myc* model described above, continued expansion is profoundly limited by ongoing apoptosis. This provides a strong selection for loss of apoptosis and, indeed, the immediate advantage these developing cells acquire from *p53* mutations is a survival advantage – that is apoptosis is the key tumor suppressor function of p53 to be circumvented. On the other hand, constitutive activation of the Ras-MAPK pathway in fibroblasts and epithelial cells can induce proliferation but, at high levels, premature senescence (Serrano *et al.*, 1997; Woods *et al.*, 1997; Lin and Lowe, 2001). In this setting, *p53* loss prevents senescence, allowing cell division to continue unabated. As Ras-expressing cells are not particularly sensitive to apoptosis, it seems likely that this increased propensity to undergo premature senescence produces selective pressure to inactivate *p53* during Ras-initiated tumorigenesis. Here, p53-mediated arrest is the key tumor suppressor function to be overcome.

In addition, the evolution of a cancer – driven by genetic or epigenetic changes that accompany progression or selected by cancer therapy – also provides strong selective pressure to disable p53 or its effector functions. As a consequence, selective pressure to thwart certain circuits of the p53 tumor suppressor network can vary during the course of tumorigenesis. How the tumor 'solves' specific problems early in tumorigenesis can influence tumor behavior later on. For example, as indicated above, the evolution of Eμ-*myc* lymphomas places strong selective pressure to disable p53-dependent apoptosis. The tumor can solve this problem through several mechanisms, for example, through inactivation of *Ink4a/ARF*, *p53*, or overexpression of Bcl-2 (Eischen *et al.*, 1999; Schmitt *et al.*, 1999, 2002a). Indeed, a substantial fraction of Eμ-*myc* lymphomas acquire spontaneous *Ink4a/ARF* or *p53* mutations during lymphoma development, and engineered lymphomas lacking *Ink4a/ARF*, *p53*, or overexpressing Bcl-2 are phenotypically indistinguishable (Eischen *et al.*, 1999; Schmitt *et al.*, 1999, 2002a). However, despite their similar overall pathology, mice bearing *Ink4a/ARF*-null tumors have a significantly better treatment prognosis than do mice with *p53*-null tumors when treated with chemo- or radiotherapy (Schmitt *et al.*, 2002b). Presumably this reflects fact that oncogenes, but not DNA damage, signal to p53 through ARF. Hence, loss of *Ink4a/ARF* or *p53* confers the same advantage to the tumor during lymphomagenesis but not during therapeutic treatment. Similarly, tumors overexpressing Bcl-2 do not undergo apoptosis in response to therapy, but instead undergo senescence. *p53* loss disables both programs, leading to a substantially worse prognosis. Thus, while *p53* mutant Eμ-*myc* lymphomas gain no immediate advantage by disabling senescence, this 'by-product' of *p53* loss produces a more drug-resistant tumor prior to therapy. Similarly, an increased propensity for genomic instability in *p53* mutant tumors, not seen in those lacking ARF or overexpressing Bcl-2, may fuel additional mutations and the evolution of drug resistance. As such, the fact that different tumors disable the p53 network at distinct points or times may contribute to the heterogeneity of human cancers.

Conclusions

As is clear from this review, p53 biology is complex. While this is not surprising given the central role of p53 in diverse stress responses, the complexity of the p53 network presents a challenge for fully understanding its biology and using this information for diagnostic, prognostic, or therapeutic purposes. However, we do know that apoptosis is a vital part of p53's tumor suppression function, as well as a great deal concerning how p53 controls the induction of apoptosis. Largely, this is through transcriptional activation of specific target genes, although evidence implicating both its transrepressive functions as well as direct effects on the mitochondria is mounting (see above). In addition, while evading p53-mediated apoptosis can be essential for tumor evolution, the manner in which a tumor does so can have an impact on tumor behavior and patient outcome.

What remains to be determined concerning the apoptotic p53 program? More upstream regulators and downstream effectors of p53 will undoubtedly be described, but the real challenge is to determine how contextual factors influence the network and how tumor heterogeneity can be understood and exploited for therapeutic purposes. Ideally, this increased understanding will permit the p53 network to be manipulated in more selective ways. Clearly, one avenue is to restore apoptosis by reintroducing a specific p53 activity, either through gene therapy or the rational design of small molecules. At the same time, the effects of p53 in normal tissues must be taken into account, including the role of p53-dependent apoptosis in producing toxic side effects from chemotherapy, as well as the potential for blocking p53-mediated apoptosis for acute or chronic diseases involving excessive cell death (Komarova and Gudkov,

2001; Tyner *et al.*, 2002). Hence, a better understanding of the p53 network may allow for custom-tailored cancer therapy, reduced therapy-induced side effects, and the ability to affect the progression of a variety of other degenerative diseases.

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Review

Dissecting p53-dependent apoptosis

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Abstract

The complexity of the p53 protein, coupled with the vast cellular responses to p53, is simply astonishing. As new isoforms, functional domains and protein–protein interactions are described; each morsel of information forces us to think (and re-think) about how it 'fits' into the current p53 paradigm. One aspect of p53 signaling that is under refinement is the mechanism(s) leading to apoptosis. Here we discuss what is known about p53-induced apoptosis, what proteins and protein–protein interactions are responsible for regulating apoptosis, how can this cascade be genetically dissected, and what pharmacological tools are available to modulate p53-dependent apoptosis. While everything may not comfortably fit into our understanding of p53, all of these data will certainly broaden our viewpoint on the complexity and significance of the p53-induced apoptotic pathway. Here, our discussion is primarily focused on the works presented at the 12th International p53 Workshop, except where appropriate background is required.

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Keywords: apoptosis; BCL-2 proteins; p53

Abbreviations: APAF1, apoptotic protease activating factor 1; ASPP, ankyrin repeat, SH3 domain and proline-rich domain containing proteins/apoptosis stimulating proteins of p53; BAK, Bcl-2 antagonist/killer; BAX, Bcl-2-associated X protein; BH3, Bcl-2-homology domain-3; BID, BH3-interacting-domain; *ced-3*, *Caenorhabditis elegans* cell-death abnormality-3; C9DN, dominant-negative caspase-9; CHC, clathrin heavy chain; FADD, FAS-associated death domain; HIPK2, homeodomain-interacting protein kinase 2; iASPP, inhibitor of ASPP1/2; ICE, interleukin-1 converting enzyme; MCL-1, myeloid cell leukemia-1; MOMP, mitochondrial outer-membrane permeabilization; $p53^{QS}$, L25Q, W26S *Trp53* knock-in; *PIDD*, *p53-indicible death domain*; PRIMA-1, p53 reactivation and induction of massive apoptosis; PUMA, p53-upregulated modulator of apoptosis; RITA, reactivation of p53 and induction of tumor cell apoptosis; tBID, truncated BID; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TRADD, TNFR1-associated death domain; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; YB1, Y-box binding protein-1

Basic Principles of Apoptosis

Apoptosis is a genetically controlled event with roles in tissue development,[1] homeostasis and disease.[2] It is defined by a pattern of molecular and morphological changes that result in the packaging and removal of the dying cell. Cells committed to die via apoptosis following developmental cues, stress or infection are removed by phagocytes to prevent a host immune response. Central to our understanding of the mechanism of apoptosis is the induction of caspase activity. Caspases are a family of cysteine proteases that orchestrate the dismantling and clearance of the apoptotic cell. Since the discovery that the *Caenorhabditis elegans* cell-death abnormality-3 (*ced-3*) gene product, which is required for apoptosis in nematodes, is homologous to the cysteine protease ICE (interleukin-1 converting enzyme), it has been recognized that caspases are required for the initiation and execution of apoptosis. There are two general signaling pathways that trigger apoptosis, the differences between these pathways dictate how the death signal is transduced, and thus, how the caspases become activated (Figure 1).

The intrinsic pathway (engaged by stresses such as DNA damage or hypoxia) is engaged by the transcriptional or post-translational regulation of Bcl-2 proteins that directly impact on mitochondrial outer-membrane permeabilization (MOMP).[3] MOMP occurs through the action of pro-apoptotic multi-domain members such as Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer (Bak); these proteins oligomerize through direct or indirect activation by the Bcl-2-homology domain-3 (BH3)-only, pro-apoptotic, Bcl-2 family members. After MOMP happens, cytochrome *c* is released from the mitochondrial intermembrane space, which causes APAF1 (apoptotic protease activating factor 1) oligomerization resulting in apoptosome formation. This complex, in turn, recruits and activates procaspase-9, which then activates executioner caspases-3 and -7. These caspases are responsible for the apoptotic hallmarks, such as chromatin condensation, plasma membrane asymmetry and cellular blebbing.

Death-receptor ligation by death ligands is responsible for the induction of the extrinsic pathway.[4] For example, tumor necrosis factor (TNF) binds to its death receptor, TNFR1, which causes the recruitment of adaptor molecules, TRADD (TNFR1-associated death domain) and FADD (FAS-associated death domain), and the binding and activation of procaspase-8 molecules that are brought together by TRADD and FADD. Once caspase-8 is active, it can activate executioner caspases. Some cells do not die in response to the extrinsic pathway alone and require an amplification step that is induced by caspase-8. In this situation, another target

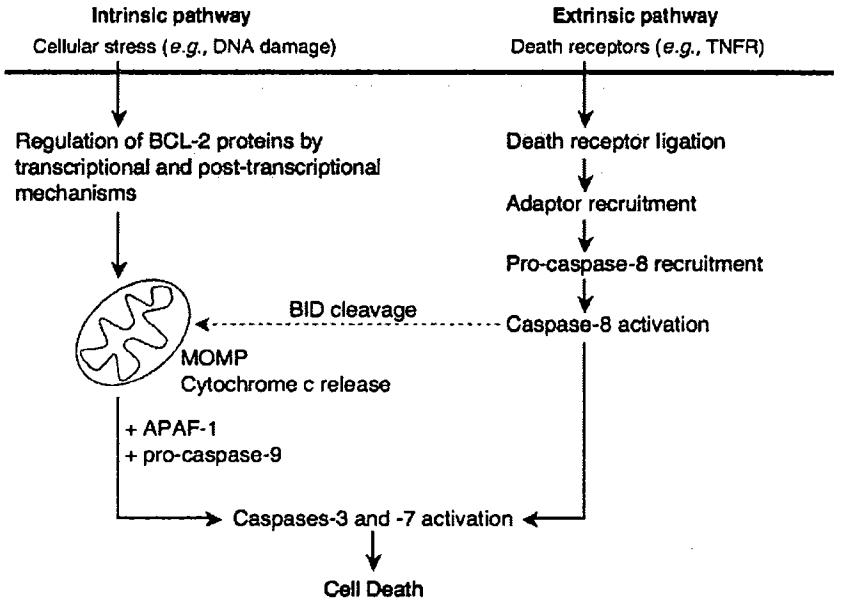

Figure 1 A comparison of the intrinsic and extrinsic apoptosis cascades. The intrinsic pathway is engaged by cellular stresses, such as DNA damage or growth factor withdrawal, and directly impacts on the Bcl-2 family of pro- and anti-apoptotic proteins. Members of this protein family can be transcriptionally-induced, transcriptionally-repressed or subjected to post-translational modifications that act to repress or enhance function. Once the appropriate repertoire of Bcl-2 proteins have been engaged, they elicit mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release and APAF-1-dependent pro-caspase-9 activation. Executioner caspases-3 and -7 are then activated by caspase-9-dependent cleavage. The extrinsic pathway requires the ligation of death receptors by death ligands, which results in the assembly of adaptor molecules and pro-caspase-8 activation. Again, executioner caspases-3 and -7 are then activated by caspase-8. Bid can also be cleaved (and activated) by caspase-8 leading to co-engagement of the intrinsic pathway

of caspase-8 is the BH3-only protein, Bid (BH3-interacting-domain death agonist). Caspase-8 cleaved Bid (tBid-truncated) is then able to directly activate pro-apoptotic multi-domain proteins to induce MOMP, so this co-engages the intrinsic pathway.

Unifying p53 and Apoptosis: What Do We Know?

Cells that are insulted by oncogene expression, DNA damage or other forms of stress stabilize the p53 protein by phosphorylation or other modifications.[5,6] Stabilized p53 accumulates in the nucleus to regulate the expression of numerous pro-apoptotic genes (e.g., *BAX, NOXA, PUMA, BID, CD95, APAF-1, DR5, p53AIP1*).[7–14] Each of these genes when silenced or removed from a particular model system produced partial resistance to p53-induced apoptosis. Most likely, these genes govern the decision to live or die based on the cell type investigated and the applied death stimulus (meaning, each gene contributes to certain death pathways, but not all). While p53 functions as a transcription factor in the nucleus (there is certainly no doubt to this ability) it also possesses an extranuclear function to directly bind anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL) and activate pro-apoptotic multi-domain Bcl-2 proteins (Bax and Bak) to regulate MOMP.[15] Years of debate over the importance of the extranuclear function have led several laboratories to develop elegant systems to demonstrate this 'unbelievable' function of p53, and the amount of literature based on this aspect of p53 function is rapidly growing.[15]

While we do not understand each step of p53-dependent apoptosis, we certainly know how p53-dependent apoptosis presents itself. Cells that have engaged p53-dependent apoptosis typically follow the intrinsic cell death pathway (Figure 2). This pathway is regulated by pro-apoptotic Bcl-2 proteins, as Bcl-2 or Bcl-xL overexpression blocks this form of cell death. Mitochondria are the downstream target of pro-apoptotic Bcl-2 proteins, and these proteins function to permeabilize mitochondria. The pro-apoptotic factors that are released from mitochondria then activate caspases that produce the hallmarks of apoptosis (e.g., DNA laddering, asymmetry of the plasma membrane). Since caspases are responsible for dismantling the insulted cell, p53-dependent apoptosis can be postponed by the addition of caspase inhibitors; but because mitochondria are permeabilized and ATP production soon wanes, death is inevitable.

Current studies focus on linking the functions of p53 to the apoptotic cascade by integrating all of p53's pro-apoptotic abilities, along with determining which proteins are responsible for generating those signals. While we don't have every piece of the cascade determined, our understanding of the p53-dependent mechanism(s) of apoptosis is becoming clearer.

Murine Models of p53-Induced Apoptosis: Defining the Elusive?

The numerous effects of p53 stabilization on maintaining tissue homeostasis and preventing aberrant cells from becoming tumors highlights the importance of understanding

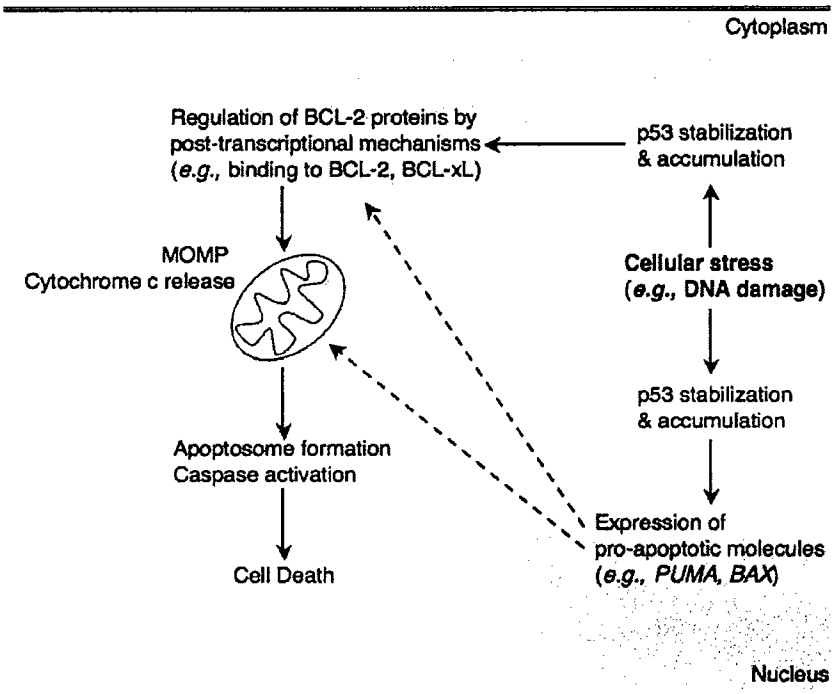

Figure 2 How p53 engages the apoptotic signal. Following cellular stress, p53 is subjected to numerous post-translational modifications that result in p53's stabilization and accumulation in the nucleus and cytoplasm. In the nucleus, p53 directly regulates the expression of numerous pro-apoptotic molecules, which are necessary for steps either directly before MOMP (e.g., *BAX*) or may potentially directly induce MOMP (e.g., *PUMA, NOXA, BID*). In the cytoplasm, p53 associates with Bcl-2 or Bcl-xL, which can release sequestered BH3-only proteins, or this interaction can inhibit p53's ability to directly activate pro-apoptotic Bcl-2 multi-domain proteins (i.e., Bax and Bak). Once MOMP occurs, apoptosome formation is initiated and the cell death cascade is engaged

which functions (or combination of functions) of p53 are essential to its tumor suppressor activity (see review by Johnson and Attardi in this issue for a detailed discussion of this topic). The earliest animal models that examined p53 function *in vivo* focused on genetically disrupting the *Trp53* gene, and these models defined the requirement for p53 function in numerous tissues, as without *Trp53*, various tumors rapidly killed p53-deficient animals.[16,17] Mice deficient in one or both *Trp53* alleles were susceptible to a spectrum of tumor types including brain and lung, lymphomas and sarcomas, and also exhibited resistance to several inducers of DNA damage.[17,18]

More recently, several groups have honed genetic approaches to directly test p53 mutations similar to those found in Li–Fraumeni Syndrome patients (by creating a murine knock-in of the mutation) to better understand the biology of naturally occurring, disease-causing p53 mutations.[19,20] The p53 point mutations selected were $Trp53^{R172H}$ (corresponding to $Trp53^{R175H}$ in humans) and $Trp53^{R270H}$ (corresponding to $Trp53R^{273H}$ in humans), which are structural and DNA contact mutants, respectively. The phenotypes of the $Trp53^{R172H/+}$ and $Trp53^{R270H/+}$ knock-in animals demonstrated that there are dramatic differences compared to $Trp53^{+/-}$. One impressive difference is the tumor profile that developed in these models. A distinct set of tumors, such as B-cell lymphomas occurred in both the $Trp53^{R172H/+}$ and $Trp53^{R270H/+}$ animals, and a marked increase of carcinomas, adenomas, hemangiomas, and osteocarcomas compared to $Trp53^{+/-}$. Also striking, is that $Trp53^{R172H/+}$ tumors demonstrated a greater propensity to metastasize, which was corroborated in a similar study. The proposed mechanisms responsible for these differences center on mutant forms of p53 acquiring a 'gain of function' to cooperate with $p53^{wt}$ and alter, perhaps, protein–protein interactions between p53 and it's binding partners, which could lead to changes in transcriptional regulation and stability.

While the models described above support the idea that loss of p53 function is deleterious to maintaining a tumor-free state; they also suggest that it will be difficult to ascertain which function(s) of p53 is primarily responsible for maintaining tumor suppression. Even so, several elegant studies have proposed that p53-dependent apoptosis is the major function required for tumor suppression *in vivo*.[21,22] Several years ago it was shown that a decrease in p53-induced apoptosis correlated with the development of aggressive tumor emergence. A more recent study has highlighted the importance of p53's ability to induce apoptosis in preserving a tumor-free state.[22] In a model of B-cell lymphoma induced by the enforced expression of the *Eμ-myc* transgene, spontaneous *Trp53* mutations arose; and likewise, a marked acceleration of tumor onset occurred when p53 function was inactivated.[23] The pressure to inactivate p53 function in this model was substantially reduced when *Eμ-myc*-expressing tumors were infected with retroviruses encoding Bcl-2 or dominant-negative caspase-9 (C9DN).[22] Both of these proteins significantly abrogate the induction of apoptosis by either directly interfering with MOMP or reducing the activation of caspases, respectively. Since neither of these proteins interfered with

the induction of cell-cycle arrest by p53, it was hypothesized that expression of Bcl-2 or C9DN is sufficient to inhibit p53-induced death, and allowed for tumor progression. Effectively, expression of Bcl-2 or C9DN phenocopied the loss of p53 in this model system, suggesting that apoptosis is the critical p53 tumor suppressor function.

Although it is clear that p53 is an important tumor suppressor, some evidence suggests that apoptosis may not be the keystone to inhibiting tumor formation. Data from the SWAP mouse (the human *Trp53* gene is knocked-into the murine *Trp53* locus) indicated that while the SWAPed p53 followed normal regulation by ultraviolet light and gamma-irradiation, in terms of phosphorylation and stabilization, it failed to induce apoptosis (and cell-cycle arrest) in several tumorigenesis models (Dmitry Bulavin, personal communication). However, the SWAPed p53 could rescue the early onset of death in $Trp53^{-/-}$ animals and substantially restore abnormal centrosomal checkpoint, indicating that non-apoptotic functions may assist in cellular stability. Interestingly, the SWAPed p53 failed to trans-activate several pro-apoptotic genes, but demonstrated normal trans-repression following ionizing irradiation; this hinted that protein–protein interactions or trans-repression may be essential for regulating tumor formation. In models of lymphomagenesis, the SWAPed p53 was often lost indicating that this form of p53 indeed had produced a selective pressure to tumor formation. Yet, it can be difficult to grasp the impact of human p53 in the context of a foreign signaling circuit, as the SWAPed p53 could possess gain- or loss-of-function behaviors in the murine milieu.

One must appreciate the complexity of p53-induced apoptosis, and understand that all p53-regulated cell death pathways are not equal. This notion is highlighted by the trans-activation-deficient double mutant L25Q, W26S *Trp53* knock-in (referred to as $p53^{QS}$).[24,25] Mouse embryonic fibroblasts derived from the $p53^{+/QS}$ animal demonstrated both compromised trans-activation of numerous p53-induced genes and cell-cycle G1 checkpoint activation. Yet, the induction of p53-regulated apoptosis was stress-dependent: DNA-damaged cell death was completely compromised, while hypoxia-initiated apoptosis was almost similar to controls. So what makes the difference? Perhaps the difference between the two stimuli is the required p53 function in each scenario. DNA-damaged induced cell death required trans-activation of pro-apoptotic genes (other than *Bax*, as this gene maintained regulation by $p53^{QS}$), while the hypoxia-induced cascade requires alternative p53 functions, such as those described as transcription-independent. Alternatively, the promoters of pro-apoptotic genes required for a hypoxia response require a different set of protein–protein interactions that are unaffected by the $p53^{QS}$ substitution, and the embryonic lethality of the $p53^{QS/QS}$ animals may support this hypothesis.

The significance of p53-mediated apoptosis in maintaining a tumor-free state may be highlighted by the conserved function of p53 (and it's appropriate homologues) in non-vertebrate model systems. Specifically, the p53 protein in other animals (at least as described in *Drosophila* and *C. elegans*) is a pro-apoptotic factor required for a proper DNA-damage response that does not appear to regulate cell cycle.[26–29] Perhaps the adoption of cell-cycle regulation and senescence activities speak to the complexity of vertebrate p53 as these additional functions may contribute to regulating the DNA-damage-induced cell death response in vertebrates.

p53 and Bcl-2 Proteins: Understanding Transcriptional Targets and Protein Interactions

When p53 was described as a pro-apoptotic transcription factor, a commitment to discover the gene(s) responsible for death was imperative. Considerable effort focused on understanding which members of the pro-apoptotic Bcl-2 proteins were regulated by p53, and, not surprisingly, works describing many Bcl-2 proteins and their regulation by p53 can be found. Bax was the first pro-apoptotic Bcl-2 protein described to be upregulated by p53 stabilization following DNA damage, and others, like the BH3-only protein Bid, soon followed.[7,10] Yet, many of these pro-apoptotic molecules are either constitutively expressed in cells (e.g., Bax) or require an additional step to be active (i.e., Bid requires caspase cleavage to be pro-apoptotic). Therefore, the direct impact of exogenous overexpression to mimic p53 upregulation confers limited interpretation as this likely upsets the balance of Bcl-2 proteins rather than reflecting the physiology of each protein. A summary of the fundamental interactions between p53 and various Bcl-2 proteins is presented in Figure 3.

As the pro-apoptotic subfamily of Bcl-2 proteins has increased to include more BH3-only proteins, further evidence suggested that these proteins also exhibit marked p53 regulation. Two such p53 transcriptional targets are *NOXA* and *PUMA*, which were originally characterized as BH3-only proteins that directly induce cell death.[8,9] Following p53 stabilization, these genes are transcriptionally upregulated and the proteins were hypothesized to directly induce MOMP. Yet, the evidence that this was indeed correct was based only on overexpression of each protein in cell lines. Further studies demonstrated that the Noxa and Puma BH3 domain peptides did not directly activate recombinant Bax or permeabilize mammalian mitochondria and Puma protein could not directly induce MOMP.[30,31]

Genetic evidence from several groups, however, did propose that *NOXA* and *PUMA* were important mediators of p53-induced apoptosis.[32–34] *NOXA* -deficient mice developed normally and exhibited proper cellularity of the hemopoietic organs, but the MEFs were slightly resistant to etoposide-induced cell death.[32,33] The most dramatic results were obtained from oncogene-expressing MEFs which displayed marked resistance to p53-dependent, UV-induced apoptosis.[33] *In vivo*, the absence of *NOXA* resulted in resistance to X-ray-induced apoptosis in the jejunum and small intestinal crypts.[32] The selective resistance to p53-induced apoptosis by the loss of *NOXA* cannot be explained by the simple explanation that BH3-only proteins can substitute for each other in apoptotic pathways, but rather these proteins function in distinct signaling cascades.[30,35] Noxa is likely to participate in p53-mediated signaling, but the exact pathways and the consequences of its activity are still unresolved.

The absence of *PUMA*, however, resulted in a broader resistance to numerous p53-dependent and -independent

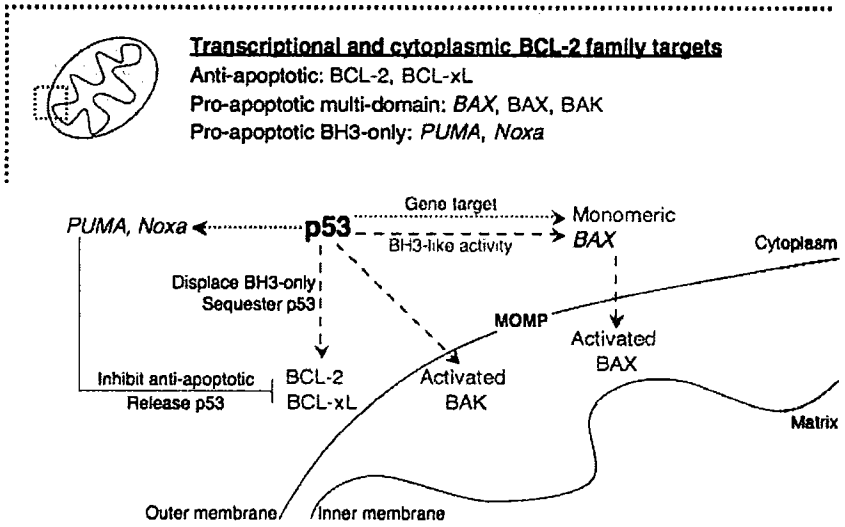

Figure 3 The relationship between p53 and Bcl-2 proteins at the mitochondrial membrane. p53 can either induce the expression of pro-apoptotic Bcl-2 proteins (e.g., *Bax, Puma, Noxa*), or it can directly regulate numerous Bcl-2 proteins in the cytoplasm. Bax that is expressed by p53 can then be activated by numerous signals, such as p53-induced BH3-only proteins (such as tBid), or by p53 itself. Bax or Bak that is activated by p53 or a BH3-only protein oligomerizes in the mitochondrial outer membrane to allow for MOMP. When Bcl-xL or Bcl-2 is bound by p53, BH3-only proteins once associated with either anti-apoptotic molecule may then be released to either directly activate a pro-apoptotic multi-domain or 'inhibit' an additional anti-apoptotic Bcl-2 protein. If nuclear p53 induces the expression of additional BH3-only proteins when cytoplasmic p53 is associated with Bcl-xL, the cell may be sensitized to numerous inducers of death. Alternatively, p53 that is sequestered by Bcl-xL may be liberated by a collateral signal, such as Puma, which frees p53 to activate Bax. Red and green arrows indicate transcriptional and cytoplasmic regulation, respectively

death stimuli *in vivo*, indicating that this protein likely functions in numerous apoptotic networks.[33,34] Like the *NOXA*$^{-/-}$ animals, *PUMA*$^{-/-}$ animals were born at the expected Mendelian frequency, appeared normal and exhibited proper cellularity of the hemopoietic organs.[33] Yet, numerous *PUMA*$^{-/-}$ cell types (e.g., thymocytes, pre-B and mature B- and T-cells) were markedly resistant to inducers of genotoxic stress (e.g., γ-irradiation); but not to the same level as *Trp53*$^{-/-}$ suggesting that the signaling cascade Puma regulates may be alternatively modulated by other proteins or mechanisms.[33] *PUMA* $^{-/-}$ MEFs expressing the *E1A* oncogene were also resistant to etoposide-induced apoptosis, similar to *NOXA*$^{-/-}$ MEFs, suggesting that functional redundancy may not be evident under this condition. Puma may also regulate p53-independent pathways because CD4^{+}8^{+} thymocytes derived from *PUMA*$^{-/-}$ animals displayed slight resistance to dexamethasone and staurosporine.[33] This suggests that Puma may participate in several death networks to sensitize cells to death stimuli that are regulated by diverse pro- and anti-apoptotic Bcl-2 proteins. Indeed, recent *in vitro* evidence supports this notion and suggests that the BH3-only proteins may function in distinct signaling networks;[30,31,35] furthermore, *in vivo* data from the *NOXA* and *PUMA* double knockout (*NOXA*$^{-/-}$/*PUMA*$^{-/-}$) present no synergistic effect in regard to DNA-damage sensitivity (Andreas Strasser, personal communication).

While the genes encoding the Bcl-2 proteins are transcriptionally regulated by p53, the influence of p53 on this protein family's function does not end in the nucleus. It is now well established that p53 also accumulates in the cytoplasm following stabilization.[36–41] The accumulation of p53 into the cytosol seems to be regulated by Mdm2, as polymorphic variants of p53 that interact better with Mdm2 accumulate more in the cytoplasm.[38] Forced localization of p53 to mitochondria can directly induce MOMP, and this is blocked by the co-expression of anti-apoptotic BCL-2 proteins.[36,38,42] This inhibition proposed that p53's ability to induce MOMP was directly regulated by pro-apoptotic Bcl-2 proteins. Likewise, several groups showed that p53 can interact with Bcl-2 and Bcl-xL, which explains how p53 can bind to mitochondria, contrary to early studies that demonstrated p53 associated with mitochondrial chaperones.[36,37] More recently, endogenous p53 has been shown to interact with Bcl-xL, and because Bcl-xL can be localized on mitochondria in several tissues, endogenous p53 can also be found on mitochondria via a Bcl-xL interaction (or, mitochondrial Bcl-2 and Mcl-1 as well).[31,43] The ability of endogenous p53 to rapidly accumulate (i.e., within 30 min of treatment) in the cytoplasm of radiosensitive organs following γ-irradiation has also been demonstrated *in vivo* in mice.[44] Most studies exploring the association of p53 with anti-apoptotic Bcl-2 proteins focused on Bcl-xL, which also led to a NMR solution structure of p53 and Bcl-xL.[45] Although, Bcl-2 almost certainly participates in this pathway as Bcl-2 overexpression also blocks p53-induced death, p53 can bind to Bcl-2, and silencing *BCL-2* by RNAi has been shown to induce p53-dependent apoptosis in certain systems.[46]

One major issue focused on determining the significance of the signal that the p53/Bcl-xL association creates within the cell. Is this the signal to initiate apoptosis, or is a collateral signal required? To examine this, a BH3-binding mutant of Bcl-xL (Bcl-xL^{G138A}) was tested for it's ability to bind p53 and either inhibit or promote cell death.[31] Interestingly, Bcl-xL^{G138A} efficiently bound p53 and inhibited p53-induced death. Moreover, when *BCL-XL* $^{-/-}$ MEFs were reconstituted to stably express Bcl-xL^{G138A} p53-induced apoptosis was also inhibited. This result led the investigators to hypothesize that the binding of p53 to Bcl-xL was not the death signal, and

perhaps required an additional signal, such as a p53-regulated BH3-only protein (as it could then bind to Bcl-xL), to promote apoptosis. Furthermore, keeping in mind the requirement for Puma in p53-mediated death, it was speculated that perhaps Puma released p53 from Bcl-xL. Indeed, the Puma BH3 domain peptide and protein acted very specifically to disrupt the p53/Bcl-xL association, and cells that expressed Bcl-xL^{G138A}, while were efficient at holding p53 inactive, failed to undergo cell death because Puma could not bind to Bcl-xL^{G138A}.[31]

However, what role does p53 play once liberated from Bcl-xL? Data suggest that p53 can act similarly to an activator BH3 protein (e.g., tBid or Bim) to directly induce the oligomerization of pro-apoptotic multi-domain Bcl-2 proteins; this function of p53 has been described for both Bak and Bax.[37,43] The addition of p53 to isolated mitochondria caused MOMP and the release of cytochrome *c*. Furthermore, in a liposome-based system designed to faithfully mimic permeabilization of the mitochondrial outer membrane, p53 functioned similar to tBid to induce the oligomization and pore-forming activity of purified, recombinant Bax.[37,47] It is worthy to note that the binding of p53 to Bcl-xL (which would repress Bcl-xL's anti-apoptotic function) may also serve to sensitize cells to further stresses that would result in BH3-only protein activation (as they would not be sequestered by p53-bound Bcl-xL).

The domains of p53 responsible for the interaction with Bcl-xL and subsequent MOMP are still not fully understood. Evidence suggests that both the DNA-binding domain (amino acids ~100–300) and the proline-rich domain (amino acids ~60–90) are essential for these interactions and cell death.[36,37,45,48] Interestingly, short peptides derived from the core domain of p53 exhibited the ability to activate multi-domains, but further work is required (Maureen Murphy, personal communication). The ability of p53 to translocate to the cytoplasm and mitochondria is regulated by MDM2 binding, and it has been shown that polymorphic variants of p53 possess distinct pro-apoptotic potentials based on their ability to be translocated out of the nucleus,[38]

The interactions described above are relatively recent observations, and the complexity of how other proteins that bind p53 and Bcl-2 proteins, including the myriad post-translational modifications each protein may demonstrate (e.g., ASPPs), impact on p53's pro-apoptotic function have not yet been elucidated.

Making the Decision to Die: Regulation of p53-dependent Apoptosis

If we consider that MOMP is central to the initiation of apoptosis, then the actual signaling mechanism(s) of p53-induced apoptosis is what lies between p53 stabilization and permeabilization of mitochondria (as the permeabilization of mitochondrial membranes, cytochrome *c* release and caspase activation are not unique to the p53-dependent pathway). In general, many of the proteins shown to promote or inhibit p53-dependent pro-apoptotic signaling have been only described to regulate p53's nuclear activity, that is, p53-dependent gene expression. Yet, as the extranuclear functions of p53 are being elucidated, several of these proteins may directly regulate p53 in the cytoplasm. Independent of cellular localization, apoptotic signaling is a very tangled network; the modulators of p53-dependent apoptosis are numerous and are themselves subject to regulation. As this area of research is so immense, this section will only highlight the topics discussed at the p53 workshop (Figure 4).

Once p53 is stabilized and accumulates in the nucleus, it must target a specific repertoire of pro-apoptotic genes to induce cell death. To achieve this, p53 either becomes post-translationally modified to recognize specific promoters or it cooperates with transcriptional regulators that deliver p53 to the appropriate site (most likely, both happen). Commonly, p53 induces the upregulation of pro-apoptotic genes, such as *BAX* and *PUMA*,[7,9] yet under conditions where cellular machinery may be compromised, for example, hypoxia, p53 can repress the expression of anti-apoptotic genes, such as

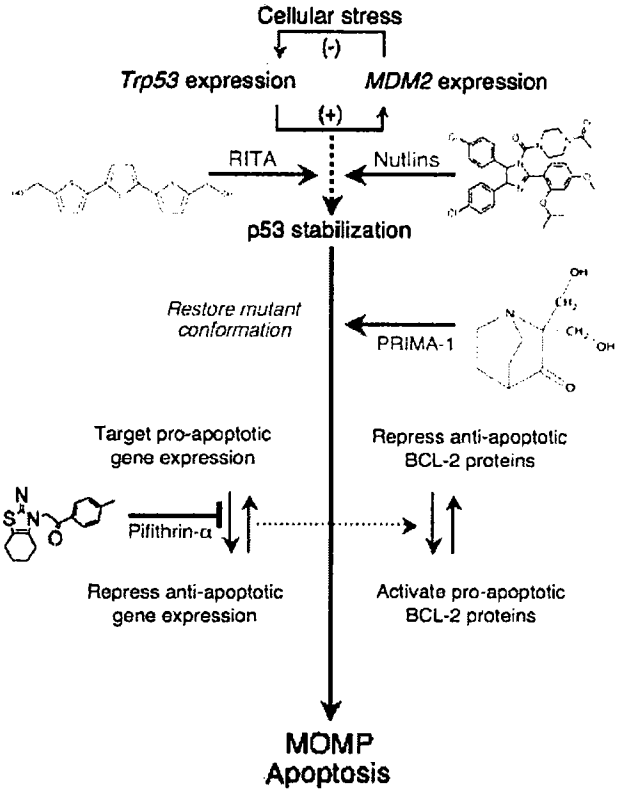

Figure 4 Cellular and pharmacological regulators of the p53-dependent apoptotic pathway. Understanding what is between p53 stabilization and MOMP/apoptosis is not well known. Most distal to the death signal is the relationship between p53 and MDM2. Until the interactions between these proteins are disrupted, no p53 signal can be induced. Data suggest that various proteins (YB1, CHC, ASPP1/2) cooperate with p53 to appropriately target pro-apoptotic genes and dictate the decision to die. Likewise, proteins like Pidd contribute in the p53 signaling cascade by bridging additional pathways to ensure death. Already mentioned in Figures 2–3 are the important interactions between p53 and Bcl-2 proteins. The blue dotted arrow indicates potential interactions with the nuclear and cytoplasmic functions of p53. The indicated small molecules demonstrate the different levels of p53 signaling that are currently regulated by pharmacological means. The interactions between p53 and Mdm2 (RITA and the Nutlins), altering mutant forms of p53 to behave like wild-type p53 (PRIMA-1) and inhibitors to p53-induced gene regulation (pifithrin-α) all hold promise, at least in theory, as 'druggable targets' that will hopefully benefit those receiving anti-tumor therapies

survivin which may promote caspase activation.[49,50] The function of p53 to repress anti-apoptotic genes targets numerous gene families, such as *BCL-2*; loss of *BCL-2*, *BCL-xL* or *MCL-1* expression would sensitize a cell apoptosis by de-repressing activated BH3-only proteins (e.g., Bim). Another scenario demonstrating the importance of repression in p53-induced death is highlighted by recent evidence that wild-type *Trp53*-expressing thyroid papillary carcinomas[51] fail to induce p53-dependent apoptosis owing to a loss of *Galectin-3* downregulation. This is perhaps owing to the lack of coordinated p53 and homeodomain-interacting protein kinase 2 (HIPK2) trans-repression of *Galectin-3*, an anti-apoptotic factor, potentially by a loss of heterozygosity for the *HIPK2* gene (Silvia Soddu, personal communication).

As mentioned earlier, p53 also coordinates with other factors to selectively target pro-apoptotic gene expression. Y-box binding protein-1 (YB1) was recently shown to block p53-dependent apoptosis, and not cell-cycle arrest or *MDM2* activity, by specifically abrogating *BAX* expression; these data support the notion that YB1 expression is associated with poor prognosis.[52] On the contrary, clathrin heavy chain (CHC), has been described to interact with p53 and enhance p53 reporter activity and apoptosis (Yoichi Taya, personal communication). Yet, CHC is a well-described cytosolic protein that regulates vesicle transport during endocytosis. Even though CHC has been shown to interact with p53 on p53 response elements by chromatin immunoprecipitation analysis *in vitro*, CHC may potentially regulate p53's cytoplasmic function as well.

As the transcriptional regulation of Bcl-2 proteins alone may not be sufficient to induce MOMP under all circumstances, p53 also induces the genes for pro-apoptotic proteins that may link intrinsic and extrinsic apoptotic programs. One example is *p53-indicible death domain* (*PIDD*), the protein of which contains a series of seven leucine rich repeats and a death domain.[53,54] These motifs are generally responsible for protein–protein interactions and transmit a cell death signal. Exogenous *PIDD* has been shown to induce apoptosis, whereas the loss of *PIDD* expression abrogated p53-dependent death. The mechanism of Pidd-induced death is not well established, but may involve caspases-2, -8, APAF-1 (apoptotic protease activation factor-1), FADD (Fas-associated death domain) and RAIDD (RIP-associated ICH-1/CED-3 homologous protein with a death domain).[53] It is described that PIDD, RAIDD and caspase-2 association resulted in caspase-2 activation,[55] but the impact of caspase-2 in p53-induced apoptosis is not concrete, as *CASPASE-2* deficiency does not abrogate p53 responses *in vivo*.

A promising area of study involves the family of ASPP (ankyrin repeat, SH3 domain and proline-rich domain containing proteins/apoptosis stimulating proteins of p53) proteins that are comprised of both positive (ASPP1 and ASPP2) and negative effectors (iASPP, an ASPP1/2 inhibitor)[56] (see review by Braithwaite *et al.* for further discussions). ASPP1 or ASPP2 coordinated with p53 to increase p53-dependent apoptosis, but they have no ability to enhance p53-dependent cell-cycle arrest.[57] In contrast, iASPP specifically inhibited p53-dependent apoptosis and cooperated with Ras, E1A or E7 to transform cells *in vitro*.[58] The mechanism of ASPP1/2 action was suggested to enhance the ability of p53 to trans-activate pro-apoptotic promoters, and iASPP could block this effect (interestingly, anti-sense iASPP promoted p53-dependent apoptosis, suggesting this mechanism is constitutively engaged). Yet, it is fairly common that p53 target genes (e.g., cell cycle and apoptosis) are simultaneously induced, and therefore the mechanism by which ASPP1/2 specifically enhanced pro-apoptotic promoter activity is not known. ASPP2, however, can also associate with numerous other proteins, such as Bcl-2.[59] The result of this interaction is hypothesized to inhibit Bcl-2 function and promote p53-dependent apoptosis (although loss of Bcl-2 alone does not do this). One additional possibility is perhaps that the Bcl-2/ASPP2 interaction regulates the cytoplasmic localization and activity of p53.

Waking up the Guardian: p53-induced Apoptosis as a Drug Target

Evidence that p53 is a promising pharmacological target (see review by Klaus Wiman in this issue for a detailed discussion of this topic) surfaced when peptides derived from the C-terminal region of p53 restored mutant p53 function and allowed for p53-dependent apoptosis.[60] Small molecules that corrected the conformation of mutant p53 soon followed, but the efficiency and required doses were not suitable for clinical use.[61] This was potentially due to the drug having to remodel the p53 protein after translation. However, these drugs rapidly evolved and another drug that could reactivate mutant p53 (both DNA contact and structural mutants – by restoring the wild-type conformation to the DNA-binding domain) to regulate p53-dependent gene expression and apoptosis was described. That drug, PRIMA-1 (p53 reactivation and induction of massive apoptosis) was hypothesized to bind nascent mutant p53 protein, rather than having to restore function to protein already folded, thus lowering the required dose (an additional drug, MIRA-1 has also been described to provoke a wild-type conformation in mutant p53 species) (Figure 4).[62] The mechanism of action for each of these drugs has not been elucidated beyond the requirement for mutant p53 and its ability to bind to p53 response elements. However, we can extend the data mentioned in earlier sections (e.g., the treated mutant p53 protein can now trans-activate the appropriate pro-apoptotic genes, bind Bcl-2 proteins, induce MOMP and caspase activation) to grasp how these molecules trigger cell death. Yet, since cells that harbor mutant p53 often exhibit marked hyper-expression and mis-localization of p53, the actual mechanism of action cannot be assumed. These caveats may, however, contribute to the observed death in tumor models, as the drugs appear to induce apoptosis in the absence of genotoxic stress (which would normally be required to stabilize the p53 protein).

The requirement for genotoxic stress or other p53-stabilizing stimuli can also be overcome by inhibiting the interaction between p53 and Mdm2, thus allowing p53 to accumulate (as the *Trp53* promoter is almost constitutively active) to promote apoptosis. One molecule, RITA (reactivation of p53 and induction of tumor cell apoptosis), was described to induce the expression of p53 target genes and marked apoptosis in wild-type p53-expressing cell lines and could coordinate an

anti-tumor response upon oncogene expression.[63] RITA functioned by abrogating the p53/Mdm2 · MDM2 interaction via binding the N-terminus of p53. A family of small molecules aimed at targeting the p53 binding domain of Mdm2, the Nutlins, were also able to induce robust p53-dependent apoptosis in tumor xenograft models.[64] Again, this class of molecules that target the p53/Mdm2 · interaction display pro-apoptotic function due to the activities of p53 described earlier (if these drugs are truly specific to the p53 pathway). This is exemplified by chromatin condensation detected by TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) staining and nuclear morphology.

These small molecules are also attractive because they can engage p53 activity in the presence of hyper-expressed Mdm2 or other situations where the p53/Mdm2 · interaction is pathologically promoted.[65,66] The Nutlins, in particular, provide both *in vitro* and *in vivo* 'proof-of-principle' evidence that the p53/Mdm2 · interface is a key drug target. As a note, an inhibitor of p53-induced apoptosis has also been described, pifithrin-α.[67] This drug may protect non-tumorigenic tissues from the undesirable side effects of cancer treatments.

Conclusion

There are a few messages that emerge from our understanding of p53-induced apoptosis. First, the ability of p53 to trigger cell death is a key tumor suppressor activity. Second, the cellular signaling cascades leading to p53-dependent apoptosis are numerous, perhaps specific to the inducer, and probably not linear (i.e., one p53 trans-activated gene may not be responsible for the entire p53 death circuit). Finally, the p53-regulated apoptotic pathway can be pharmacologically modulated to hopefully treat cancer patients. Taking these three ideas into consideration will help us to reach a better understanding of the relevance of p53 and cell death in both principle and practice.

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COOH-Terminal Domain of p53 Modulates p53-mediated Transcriptional Transactivation, Cell Growth, and Apoptosis[1]

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ABSTRACT

The tumor suppressor protein p53 contributes to the control of cell cycle checkpoints and stress-induced apoptosis and is frequently mutated in many different types of human cancers. The COOH terminus of p53 modulates the transcriptional and apoptotic activities of the protein. Although COOH-terminal mutants of p53 are uncommon, we proposed that these p53 mutants nevertheless contributed to the selective clonal expansion of the cancer cells. Therefore, we analyzed the tumor-derived p53 COOH-terminal domain (CTD) mutants (352D/H, 356G/W, 342-stop, 360-del, and 387-del) functionally. The results have revealed that all mutants have impaired apoptotic activity when compared with wild-type p53. However, some of these mutants still transcriptionally transactivate *p21*$^{Waf1/Cip1}$ and inhibit cell growth. Interestingly, of the tumor-derived CTD mutants, oligomerization-defective mutant 342-stop was the only one that did not exhibit sequence-specific DNA binding or failed to transactivate *p21*$^{Waf1/Cip1}$, *Bax*, and *IGF-BP3* transcriptionally. The failure to inhibit cell growth by this tumor-derived CTD mutant supports the hypothesis that p53 sequence-specific transcriptional transactivity to *p21*$^{Waf1/Cip1}$ is correlated with induction of cell cycle arrest and that the p53 transcriptional transactivity requires oligomerization of the p53 protein. These and other data indicate that the CTD of p53 is an important component of p53-mediated apoptosis and cell growth arrest and that inactivation of the apoptotic function, but not the inhibition of growth, is an important step during human tumorigenesis.

INTRODUCTION

Two major functions of p53 are the negative regulation of cell growth and the induction of apoptosis. Cells lacking functional p53 fail to arrest in G_1 following γ-irradiation (1), whereas introduction of wild-type p53 leads to G_1 arrest in either p53-null or p53-mutated cells (2–4). Growth arrest is controlled principally by transcriptional modulation of p53-transactivated genes, such as *p21*$^{Waf1/Cip1}$ (an inhibitor of cyclin-dependent-kinase) and *GADD45* (a growth arrest and DNA damage responsive gene; Refs. 5–7). Apoptosis can be triggered by overexpression of transfected wild-type p53 in p53-null tumor cells (8). Thymocytes derived from p53 knock-out (p53 −/−) mice are resistant to radiation- and drug-induced cell death (9). Some tumor-derived p53 mutants display defects in apoptotic activity and still retain growth suppression (10). These data indicate that the distinct functions of p53 are mediated via specific pathways.

p53 has multiple, unique, functional domains. These include the NH_2-terminal transactivation domain, the sequence-specific DNA-binding domain that is necessary for the transcription transactivation, and the CTD.[3] The CTD of p53 has drawn considerable attention in recent years based on its multiple functions (11–16). The COOH terminus of p53, comprising amino acids 311–393, can be subdivided into at least two structural determinants: the oligomerization domain (amino acids 319–360) and an adjacent basic region (amino acids 363–393), which also has been referred to as an apoptotic domain (17); a transcriptional regulatory domain (18); or a DNA damage recognition domain (14, 19). The oligomerization domain is required for the p53 tertiary structure, which regulates transcriptional transactivity by altering the conformation of the protein (20, 21). Modifications of the negative transcriptional regulatory domain of p53 CTD by phosphorylation, acetylation, deletion, or antibody binding have been shown to play important roles in the regulation of p53 sequence-specific binding (12, 16, 22–25). Wild-type p53 can transactivate genes transcriptionally through binding to specific DNA sequences in the promoter regions of genes involved in cell cycle control and apoptosis, *e.g.*, *p21*$^{Waf1/Cip1}$, *Bax*, and *IGF-BP3* (5, 26, 27). The COOH terminus seems to exert a negative effect on the DNA-binding activity of the core p53 domain (22, 28). Deletion of the last 30 amino acids was shown to abolish this repressor activity (22, 28). Furthermore, it was found that an alternative COOH-terminal spliced p53 exhibited an enhanced DNA-binding activity (29).

p53 mediates apoptosis by both transcription-dependent and transcription-independent pathways (7, 17, 26, 30–32). The contribution of sequence-specific transcriptional activation to the induction of apoptosis is less clear than the induction of cell cycle arrest. The CTD is involved in the apoptotic function of p53 by protein-protein interactions (17). The CTD of p53 can bind directly to XPB and XPD, inducing apoptosis (17, 33). The hepatitis B virus X protein binds to CTD and inhibits p53-mediated apoptosis (34). The CTD of p53 can bind single strands of DNA and DNA ends that are likely to be present in irradiated cells, implicating that CTD is responsible for sensing these types of DNA damage (17, 35).

We have reported previously that overexpression of wild-type p53 in primary human fibroblasts induced apoptosis and that deletion of the last 40 amino acids at the CTD abolished p53-mediated apoptosis completely (17). These data indicate that the CTD may contribute directly to apoptosis. Analysis of the p53 mutation spectrum (36, 37) indicates that ~5–15% of the p53 mutations occur in the CTD during human tumorigenesis. Most of the somatic mutations in the p53 CTD are either nonsense mutations or lead to frameshifts, resulting in a truncated protein (Fig. 1*A*). To further examine the biological functions of the p53 CTD, we investigated tumor-derived p53 CTD mutants that were presumably selected during the clonal evolution of human cancers. The results indicate that the CTD of p53 regulates transcriptional activity and sequence-specific DNA binding and modulates the apoptotic process. The CTD of p53 also is involved in G_1 arrest and cell growth inhibition, is closely correlated with transcriptional activation of *p21*$^{Waf1/Cip1}$, and can be independent of p53-mediated apoptosis.

MATERIALS AND METHODS

p53 Mutagenesis and Vectors. Five tumor-derived p53 CTD mutants: 352D/H, 356G/W, 342-stop (342R/Stop), 360-del (360 1-bp deletion), and 387-del (387 19-bp deletion; Refs. 38–42) were constructed by site-directed mutagenesis. The mutations were verified by DNA sequencing. Wild-type p53

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[3] The abbreviations used are: CTD, COOH-terminal domain; LFS, Li-Fraumeni syndrome; EMSA, electrophoretic mobility shift assay; TFIID, transcription factor IID.

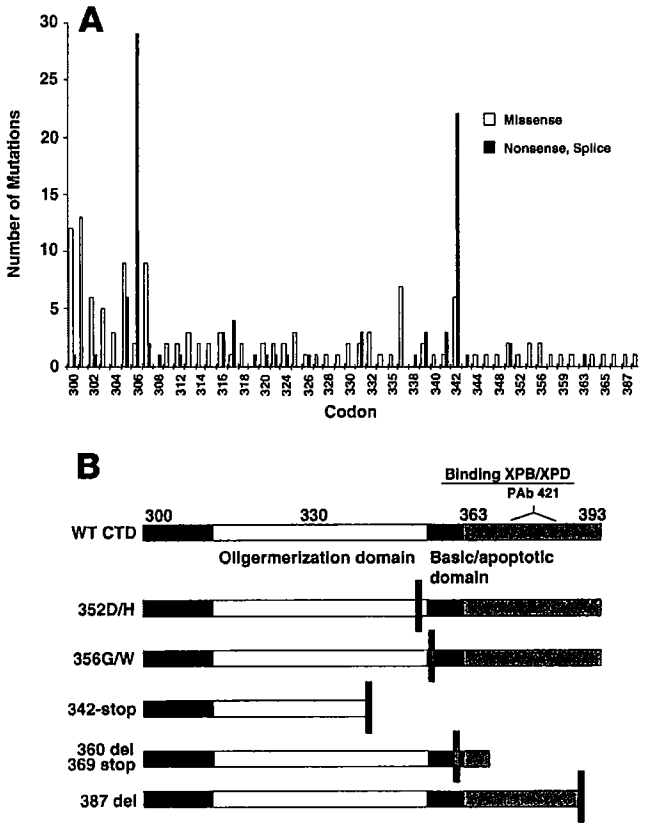

Fig. 1. Mutational spectrum found in p53 CTD (*A*) and p53 CTD and tumor-derived p53 CTD mutants used in this study (*B*). p53 CTD can be subdivided into three regions: flexible linker (residues 300–320), oligomerization domain (320–360), and basic domain (363–393). Mutant *352D/H* was derived from an esophageal squamous cell carcinoma, *356G/W* from an endometrial carcinoma of uterus, and *387-del* from a bladder carcinoma. The *342-stop* mutation occurred in several different cancer types. A 1-bp deletion at 360 was found in hereditary, nonpolyposis colorectal cancer, which created a new stop codon at residue 369, altered seven amino acids, and truncated the last 25 amino acids.

and the CTD mutants were inserted into the pcDNA3 vector under the control of the cytomegalovirus early promoter.

Cell Culture, Microinjection, and Immunocytochemistry Analysis. Primary human fibroblasts and an immortalized p53-null LFS fibroblast cell line (041) derived from the skin biopsies of a patient with LFS were grown in Ham's F10 medium supplemented with 10% fetal bovine serum. Cells were seeded onto coverslips and incubated for 1–2 days prior to microinjection. pcDNA3 vectors containing wild-type p53, the CTD mutants, or *LacZ* as a negative control at a concentration of 200 ng/μl, were microinjected into the nuclei of cells, using a microcapillary glass needle. For each experiment, at least 100 cells were injected, and a typical experiment yields at least 50 positive cells for analysis. After incubation for 24 h, cells were fixed with 4% paraformaldehyde followed by methanol treatment. p53 was visualized by staining the cells with the anti-p53 polyclonal CM-1 antibody (Signet Labs, Dedham, MA), and followed by fluorescein-conjugated antirabbit IgG (Vector Labs, Burlingame, CA) as described previously (17). Nuclei were stained with 4′, 6-diamidino-2-phenylindole. Apoptotic cells that were p53-positive were scored by their smaller size, condensed chromatin, and fragmented nuclei, as compared with control cells.

Cell Culture and Reporter Gene Assay. LFS fibroblasts cultured in 12-well plates at 60% confluence were transfected transiently with 1 μg of pcDNA3 p53 expression vectors, using Lipofectin (Life Technologies, Gaithersburg, MD). The p53 transcriptional transactivities were examined with the Dual-Luciferase Reporter system (Promega, Madison, WI) by cotransfecting 0.025 μg of Renilla Luc vector SV 40 (as an internal control) and 0.5 μg of p53-responsive reporter vectors, WWP-Luc-p21, PGL3-Luc-Bax, or pUHC13–3-Luc-IGF BP3-BOX B (26, 43, 44). Five h after transfection, the medium was changed. After an additional 15 h of incubation, the cells were lysed and the lysates were collected for the activity measurement. The activity of luciferase was quantified with a Luminometer (Analytical Luminescence Lab., Ann Arbor, MI).

To evaluate the expression level of wild-type and CTD-mutated p53 after transfection, the cells were lysed in RIPA buffer [150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)]. Forty μg of total cellular protein were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a purified, biotinylated mouse monoclonal antibody that recognizes the p53 protein (Oncogene Research Products, Cambridge, MA).

Expression of the p53 Protein Constructs and EMSA. To generate p53 proteins for EMSA, 1 μg of each of the pcDNA3-p53 constructs was used for the *in vitro* transcription-translation assay, according to the procedure described by the Promega manufacturer for the TNT T7 Quick Coupled Transcription/Translation System (Promega). The DNA-binding activities of the wild-type and the CTD-mutated p53 proteins were analyzed by EMSA. The consensus sequence oligonucleotide, TCGAGAGGCATGTCTAGGCATGTCTC (44) was synthesized, prepared in double-stranded form, and end-radiolabeled with ^{32}P-ATP. Four μl of the translated protein products were mixed with 20 fmol of radiolabeled DNA, 1 μl of PAb-421 ascitic fluid (when indicated; +), 2 μg (2 μl) of poly dI-dC as nonspecific DNA, and one-half of the reaction volume of buffer (25 mM Tris-HCl, 100 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol). The reactions were incubated for 15 min on ice and for an additional 15 min at room temperature. The samples were analyzed in 4% polyacrylamide gels electrophoresed at 350 V in 0.5 Tris-borate-EDTA buffer at 4°C.

Colony Formation Assays. Both p53-null LFS fibroblast cells (041) and Hep 3B cells were cultured in EMEM medium supplemented with 10% fetal bovine serum. Five μg of pcDNA3 containing wild-type p53 or the CTD mutants were transfected into the cells at 60% confluence in 60-mm dishes. Forty-eight h after transfection, G418 was added for the selection. The cells were maintained in the G418-containing medium for 10–14 days, and the colonies were counted. The experiments were repeated at least three times. The significant difference (*P*) was tested by the Student's *t* test.

RESULTS

Induction of Apoptosis by p53 CTD Mutants. To test the biological significance of the CTD mutations selected during tumorigenesis, we investigated five tumor-derived p53 CTD mutants (Fig. 1*B*), including a mutation hotspot, 342-stop. These nonsense, missense, deletion, or frameshift mutations occurred in either the oligomerization domain or the apoptotic domain of CTD.

Consistent with our previous data, the wild-type p53 induced apoptosis in ~20% of the p53-positive cells in both primary human

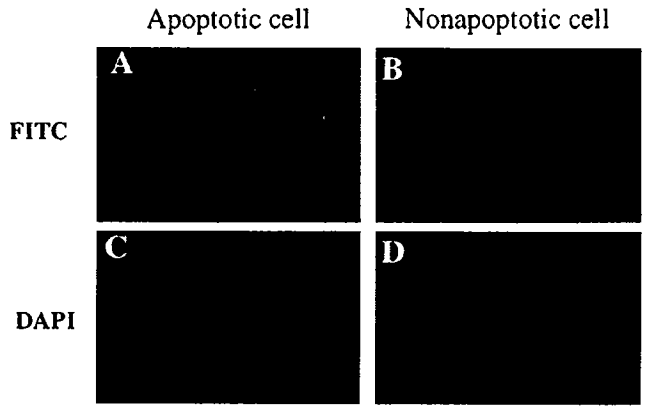

Fig. 2. Induction of apoptosis in LFS fibroblasts (p53 null) by WT-p53. p53 protein was stained with CM-1 antibody (*FITC*), and nuclei were stained with 4′,6-diamidino-2-phenylindole (*DAPI*). Apoptotic cells showed a smaller size, condensed nuclei, and chromatin when compared with the nonapoptotic cells.

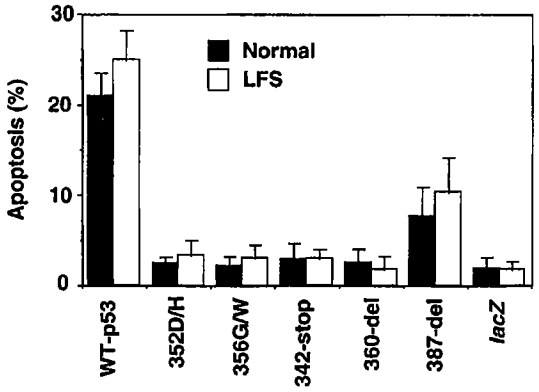

Fig. 3. Induction of apoptosis by wild-type p53 (*WT-p53*) and tumor-derived p53 CTD mutants. Apoptotic cells among p53-positive cells were scored by the morphological characteristics 24 h after microinjection. All data are an average of three independent experiments, and a total of ~200 cells were examined. *Bars*, SD.

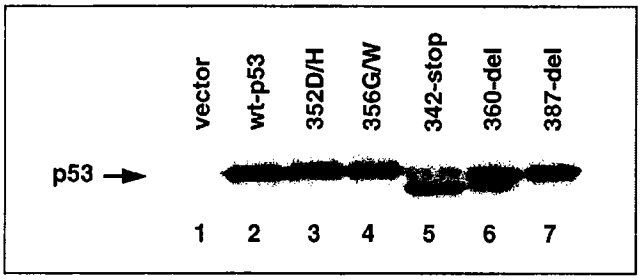

Fig. 4. Western blot of extracts from p53-null LFS fibroblasts transfected with wild-type (*wt-p53*) and CTD-mutated p53. The similar levels of p53 expression are shown. Truncated protein representing the *342-stop* mutant can be seen by smaller size.

fibroblast cells and LFS fibroblast cells (p53 null), 24 h after microinjection (Figs. 2 and 3). The cells that were microinjected successfully with either wild-type p53 or the CTD mutants showed expression of p53 by antibody CM-1 staining (Fig. 2, *A* and *B*). p53 localized mainly in both the nucleus and cytoplasm. No significant alterations of localization between wild-type p53 and CTD mutants were observed. p53 immune-positive and apoptotic cells showed fragmented nuclei and condensed chromatin (Fig. 2*C*). Interestingly, most of the p53 CTD mutants have a significant reduction in apoptotic activity that was similar to the background rate induced after microinjection of a *LacZ* expression vector (Fig. 3). The p53 CTD mutant 387-del displayed a slight reduction in apoptotic rate.

Transcriptional Transactivation Activity of p53 CTD Mutants. p53 is a DNA-binding-dependent transcriptional transactivator, and the p53 CTD is a regulator of sequence-specific DNA binding (12, 15, 22, 23, 26, 27). To test the transcriptional transactivation activity of the tumor-derived p53 CTD mutants, the expression vectors encoding wild-type p53 or the CTD mutants were transfected into p53-null LFS fibroblasts along with reporter genes containing promoter elements from genes transcriptionally regulated by p53. In the transient transfection assay, the protein expression levels of the CTD mutants were similar to that of wild-type p53 (Fig. 4). However, the effects on transcription of the reporter genes was variable. The mutant 342-stop showed an attenuated transcriptional activation of the $p21^{Waf1/Cip1}$ promoter, but the other tumor-derived p53 CTD mutants activated transcription similar to wild-type p53 (Fig. 5*A*). All of the tumor-derived p53 CTD mutants demonstrated a modest attenuated activation of the *Bax* and *IGF-BP3* promoters (Fig. 5, *B* and *C*). The mutant 342-stop showed no detectable transcriptional transactivity of these genes relative to the vector control (Fig. 5, *B* and *C*).

DNA Binding Affinity of p53 CTD Mutants. p53 sequence-specific DNA binding is required for its transcription transactivation function (26, 45). To evaluate the specific DNA-binding activity of the various COOH-terminally modified p53 mutants, the binding of p53 CTD mutant proteins to p53 consensus sites was determined by EMSA. *In vitro* translated p53 proteins are shown in Fig. 6*A*. As expected, the *in vitro* translated wild-type p53 protein does not bind to DNA spontaneously; however, this activity is enhanced by the anti-p53 antibody PAb-421 (Fig. 6*B*). The p53 CTD mutant 342-stop did not bind the p53 responsive element either in the absence or presence of PAb 421 (Fig. 6*B*). In contrast to the 342-stop, the mutant 360-del has spontaneous DNA binding activity. Neither the 352D/H mutant nor 356G/W affected the DNA binding activity of those proteins (Fig. 6*B* and Table 1).

Inhibition of Cell Growth by Wild-Type p53 and the CTD Mutants. The inhibition of cell growth and induction of cell cycle arrest by p53 depend on p53-mediated transcriptional activation (46). Transcriptional activation of $p21^{Waf1/Cip1}$ appears to contribute to cell growth inhibition (5). The 342-stop mutant, which showed no transcriptional activation from the $p21^{Waf1/Cip1}$ promoter (Fig. 5*A*), lacked the growth inhibition function in both LFS fibroblast and Hep 3B cells, whereas the other mutants retained growth suppressor activity similar to wild-type p53 in both cell

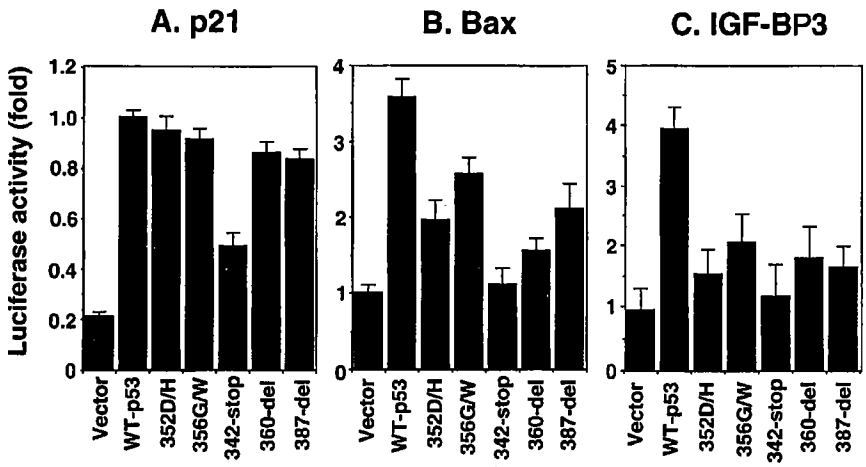

Fig. 5. Sequence-specific transcriptional activation by wild-type p53 (*WT-p53*) and the CTD mutants in LFS fibroblasts. Activities of the *p21* promoter WWP-Luc-p21 (*A*), *Bax* promoter PGL3-Luc-Bax (*B*), and *IGF-BP3* promoter pUHC13-3-Luc-IGF BP3-Box B (*C*) were detected by cotransfection of the p53 plasmid. Results represent the mean of three independent experiments; *bars*, SD.

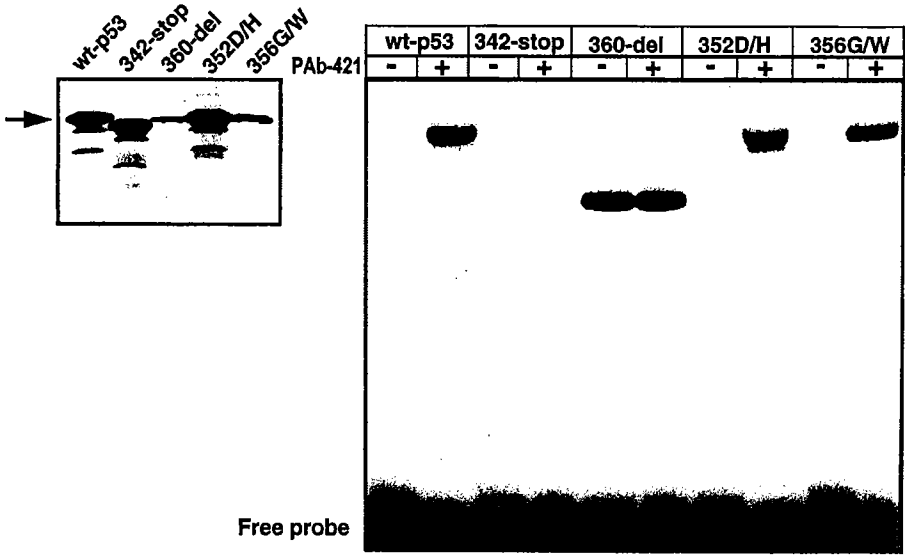

Fig. 6. *A*, p53 proteins translated *in vitro*. The different ^{35}S-methionine-labeled proteins were run in a SDS-PAGE. The *arrow* points to the p53 expected proteins. *B*, sequence-specific DNA binding affinity of *in vitro*-translated wild-type p53 (*wt-p53*) and CTD mutants. DNA-binding activities of *in vitro*-translated p53 proteins are enhanced by the *PAb-421* antibody. Oligonucleotides containing the p53 consensus binding sequence was used. *342-stop* is not able to bind the p53 responsive element, and *360-del* shows spontaneous bingeing activity with or without PAb-421 antibody.

types (Fig. 7). The effects of the tumor-derived p53 CTD mutations on various biological functions measured are summarized in Table 1. All of the tumor-derived p53 CTD mutants were defective in the induction of apoptosis. The p53 CTD mutant 342-stop with no inhibition on the cell growth showed neither transcriptional activation on target genes tested nor sequence-specific DNA binding. Each of the p53 CTD mutants 352D/H, 356G/W, 360-del, and 387-del bound to the p53 consensus sequence and retained most of its transcriptional transactivity. The p53 CTD mutant 360-del activated the sequence-specific binding to the $p21^{Waf1/Cip1}$ (data not shown) and p53 consensus binding site PG13.

DISCUSSION

p53 responds to cellular stress and maintains genomic stability (1, 47–50). However, this multifunctional property of p53 makes it difficult to determine the precise mechanism(s) by which p53 functions as a tumor suppressor. p53 may also induce apoptosis by transactivating proapoptotic genes such as *Bax* and *IGF-BP3* (26, 27) and by transrepressing antiapoptotic genes such as *bcl*-2 (51). IGF-BP3 antagonizes the activity of insulin-like growth factor 1, and a reduction in the level of the insulin-like growth factor 1 type II receptor has been shown to result in apoptosis (27). In our study, the tumor-derived p53 CTD mutants have diminished apoptotic activity and reduced transcriptional transactivity of *Bax* and *IGF-BP3*, suggesting the interactive effects of p53 transcription-dependent and transcription-independent apoptotic pathways in normal and LFS fibroblasts. We explored the significance of the p53 CTD by examining several of the biological functions of tumor-derived p53 CTD mutants. A deficiency in apoptosis was a common characteristic of all of the tumor-derived p53 CTD mutants examined. This finding is consistent with the model that apoptosis is an important function for p53-mediated tumor suppression and that loss of this function by mutation at the p53 CTD favors tumorigenicity.

Previously, we reported that the COOH-terminal alternatively spliced p53 product altered the apoptotic function at different time points (52). We showed recently that microinjection of the p53 CTD polypeptide 319–393 into normal primary human fibroblasts, mammary epithelial cells, and p53-null LFS induced apoptosis (17). p53 CTD polypeptides can also induce apoptosis in human cancer cell lines (13). These data indicate that the p53 CTD can be sufficient to induce apoptosis. The CTD of p53 binds to both cellular and oncoviral proteins that can alter p53 functions (15, 53). For example, the p53 CTD can recognize damaged DNA and may influence DNA repair mechanisms through an interaction with replication protein A and TFIIH (16, 54). TFIIH is a multiprotein complex involved in transcription (55), nucleotide excision repair (33, 56), and apoptosis (17). p53 binds to four proteins, XPD, XPB, p36, and p62, in the TFIIH complex (33, 34, 57, 58). The COOH terminus of p53 binds specifically to the DNA helicases XPB and XPD in TFIIH, which are essential for transcription and nucleotide excision repair and contribute to apoptosis (17). p53-mediated apoptosis does not require RNA and protein synthesis (59, 60), and thus supports the hypothesis that p53-dependent apoptosis can occur through a transcription-independent pathway (31, 61–63). Our data are consistent with this hypothesis, indicating that the p53 CTD contains an apoptosis domain and that its induction of apoptosis may be mediated through its binding to TFIIH and other cellular protein mechanisms.

Table 1 *Biologic effects of wild-type p53 or tumor-derived CTD mutants in human fibroblasts*

p53	Apoptosis	Growth arrest	Transcriptional activity			DNA-binding affinity
			p21	*Bax*	*IGF-BP3*	p53 consensus binding site
WT-p53[a]	++	++	++	++	++	++
352D/H	−	++	++	+	+	++
356G/W	−	++	++	+	+	++
342-stop	−	−	−	−	−	−
360-del	−	++	++	+	+	++
387-del	−/+	++	++	+	+	++

[a] WT, wild-type; ++, same level as wild-type p53; −, same level as vector only; +, reduction compared with wild-type p53; del, deletion; −/+, significant reduction compared with wild-type p53 ($P < 0.01$).

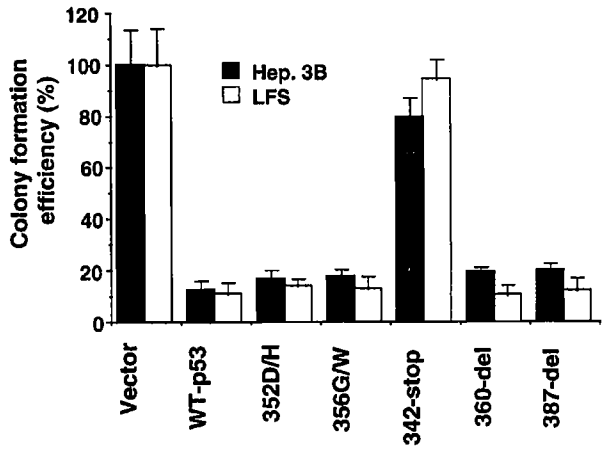

Fig. 7. Inhibition of growth of LFS fibroblasts and Hep 3B cells by wild-type p53 (*WT-p53*) and the CTD mutants. Cells were transfected with expression vectors; *colony formation efficiency* is represented by colony numbers counted after 2 weeks of G418 selection. Data represent the mean of three independent experiments; *bars*, SD.

One of the important functions of p53 is its ability to act as a transcriptional transactivator. The CTD mutant 342-stop abolished both suppression of the cell growth and transactivation of $p21^{Waf1/Cip1}$, *Bax*, and *IGF-BP3* in p53-null LFS fibroblasts. Failure to transactivate was associated with reduced ability of the mutant to bind the p53-responsive DNA sequences present in the target genes. The other tumor-derived p53 CTD mutants activated $p21^{Waf1/Cip1}$ transcription to the same extent as wild-type p53; however, the transcriptional transactivity of *Bax* and *IGF-BP3* was reduced moderately. The heterogeneity of the transcriptional transactivities on p53-responsive genes has been reported previously (45, 46, 64). These reports and the data presented here indicate that there are individual pathways to regulate different p53 downstream target genes, which are necessary for the different biological functions of p53. The lack of transcriptional transactivity by the tumor-derived mutation 342-stop indicates that the last 50 amino acids are critical for the transcriptional transactivation.

The transcriptional transactivity of p53 is dependent on the specific recognition of DNA sequences located in the promoter regions of the target genes, and p53 requires activating factors to confer effective sequence-specific DNA binding activity (22, 44, 54, 65). The CTD of p53 has been shown to be important in regulating the activity of the whole protein by modulating its sequence-specific DNA binding and transcriptional transactivity. The modulating factors include mutation, phosphorylation, acetylation, or PAb-421 binding within CTD of p53 (12, 16, 18, 22). Our data are consistent with these findings. The 360-del mutant has seven altered amino acids and a stop codon at 369. This protein has lost the PAb-421 epitope, but kept intact the oligomerization domain, and behaves as the described p53Δ30 mutant (28) or the alternatively spliced p53-encoded protein found in murine cells (29, 66). The truncated protein-product of the 342-stop mutant lost its DNA-binding activity because of the loss of the oligomerization domain. The PAb-421 antibody also has no effect due to the lack of the specific epitope. Our results support the concept that transcriptional transactivity is dependent on sequence-specific binding and that the binding affinity is regulated and governed by the p53 CTD. All mutants, except for 342-stop, retain the cell arrest function, indicating that p53 CTD is involved in the inhibition of cell growth and that this function is not associated with its induction of apoptosis. Taken together, these results are consistent with the hypothesis that a diminution of the apoptotic function of p53 is important in its tumor suppression activity and that the CTD of p53 contributes to this activity.

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